REMARKS

This preliminary amendment is being submitted to insert a claim of priority based on a foreign application into the specification. The substitute specification has been provided to correct typographical errors.

Pages 1 – 5 in the Background Art section and 29, 30 in the Technical Solution were amended to correct grammatical errors. Similar corrections were made to Examples 1 – 7. The amendments to the specification serve to merely correct grammatical errors and split lengthy sentences into several shorter ones. The amendments to the claims find basis in the claims as originally presented. More specifically, new claims 21, and 24 – 26 correspond to original claims 1 – 5 and 15; new claims 22 -26 to original claims 6 - 8; new claims 22 – 29 to original claim 15 and specification descriptions beginning in line 24 page 12, and line 22, page 16. New claim 30 correctly to original claims 10 and 11; new claims 31 – 34 correctly to original claim 9; new claims 35 and 36 correspond to original claims 12 and 13; new claims 37 and 38 correspond to original claims 17 and 18; new claims 39 - 40 correspond to the original claim 19; and the newly added claims 41 - 47 correspond to original claim 20 and specification page 15, lines 7 - 15.

No new matter has been inserted into the application by these amendments. The present application is believed to be complete in all respects and in condition for favorable consideration.

Respectfully submitted,

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Date of Deposit December 22, 2005
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Marked Up Version

THE DIMER OF CHIMERIC RECOMBINANT BINDING DOMAIN-FUNCTIONAL GROUP FUSION FORMED VIA DISULFIDE-BOND-BRIDGE AND THE PROCESSES FOR PRODUCING THE SAME.

CROSS REFERENCE TO RELATED APLICATIONS

This patent application claims the benefit of priority from Korean Patent Application No. 10-2003-0043599 filed June 30, 2003 through PCT Application Serial No. PCT/KR2004/001595 filed June 30, 2004 the contents of which are incorporated herein by reference.

Technical Field

This invention is related to the dimer of chimeric recombinant binding domain-functional group fusion([B-F fusion]$_2$) by using disulfide-bond-bridge connecting the two of a monomer of chimeric recombinant binding domain-functional group fusion(B-F fusion) and method for producing the said dimer.

Background Art

The inventive dimer of chimeric recombinant binding domain(B)-functional group(F) fusion was the first to be formed by using disulfide-bond-bridge to connect monomers having twice number of binding domain, and it has higher efficiency for targeting its functional group to the targets than the monomer and the production yield is high containing said extension peptide chain~~(LFA, lath flexible amino acid)~~.

The fusions of binding domain and heterogeneous functional group have been made of various kinds of binding domains and heterogeneous functional groups.

Antibodies are typically used as binding domains[Reference: Hall, Walter A., Immunotoxin Method and Protocols, *Methods in Molecular Biology* Vol 166,

Humana Press, Totowa, New Jersey]. Antibodies have been studied via changing their binding regions through recombination and modification while still maintaining their binding affinities and binding specificities. For example, there are scFv, pFv, dsFv, Fab, L(using one light chain), LL(using two light chains), H(using one heavy chain), HH(using two heavy chains), diabody, triabody, tetrabody, double headed antibody and others[Reference: Brinkmann, U.,*et al.*, *J. Mol. Biol.* 268, 107~117, 1997, Chaudbary, V.K., *et al.*, *Nature* 339, 394~397, 1989, Webber, K.O., *et al.*, *Mol. Immunol.* 4, 249~258, 1995,Yokota,T., *et al.*, *Cancer Res.* 52, 3402~3408, 1992, Kreitman R.J., *et al.*, *Leukemia* 7(4), 553~562, 1993, Pluckthun A. and Pack P., *Immunotechnology* 3, 83, 1997, Hollinger,P.,*et al.*, *Protein Eng.* 9, 299~305, 1996, Atwell J.,*et al.*, *Protein Eng.* 12, 597~604, 1999, Iliades P., *et al.*, *FEBS Lett.* 409, 437, 1997]. These fragments of antibody binding regions have been used independently without fusing to functional groups or used as a fusion with functional groups which provokes physiological responses to target cells to deliver the functional group specifically.

Also examples of ~~uses as~~ binding domains include many kinds of ligands or fragments which have ligand binding affinities, such as, TGF alpha, TGF beta, IL2, IL6, TNF, GMSCF. And they <u>also</u> include many kinds of ligand receptors or fragments which have receptor binding affinity, for example, TBP1, TBP2, IFN alpha or beta receptor, gonadotropin receptor and other receptors.

There are many functional groups that have been used in fusion with binding domains [Reference: Hall, Walter A., Immunotoxin Methods and Protocols, Methods in Molecular Biology Vol 166, Humana Press, Totowa, New Jersey]. For example, enzymes that have functions in prodrug transformation, material detection, decomposition, formation, proteins containing cytotoxic functional group and other functional group, organisms including the viruses for gene therapy, compounds that form cationic tail for delivering DNA, drug

compounds, liposomes for drug delivery, biosensors for detecting real time target molecule and many others are used as functional group for fusion[Reference: (Hudson,P.J., *Curr Opin Immunol* 11(5), 548~5, 1999)(Bagshawe, K.D., *et al., Curr Opin Immunol* 11(5),579~83, 1999)].

An antibody-toxin functional group fusion is a molecule which has a cytotoxic factor connected via chemical or genetic methods to an antibody as a specific cell binding-domain[Reference: Cobb, P.W., *et al., Semin Hematol* 29, 6~13, 1992]. It was expected that antibody-toxin functional group fusions would be successful in cancer treatment once —antibodies recognizing cancer cells become available.

Antibody-toxin functional group fusion in its early stages was made by connecting two proteins via protein chemical cross-linking reaction, but in accordance with the development of recombinant DNA technology, it was produced in various forms of recombinant protein through genetic fusion. The early antibody-toxin fusion(mAb-toxin) proteins made by chemical cross-linking showed a high stability in blood. They exterminated cancer cells in a clinical demonstration[Reference: Pai, L.H.,*et al., Cancer Res.* 52, 3189~93, 1992], but the damages done on the antibody during the chemical reaction and the loss of activity caused by side reactions remained as problems.

These problems were solved mostly through recombinant DNA technology. Genetic engineering allowed the production of pure and homogeneous antibody-toxin fusion molecules. It also enabled the design and production of proteins with small molecular weights [Reference: Pai, L.H.,*et al., Proc Natl Acad Sci* USA 88, 3358~3362, 1991]. For the minimal functional domain for antibody-toxin functional group fusions, the variable region of an antibody binding site(except the constant region) and the cytotoxic domain of a toxinwas used(excluding the cell-binding domain of the toxin)[Reference: Kondo, T., *et al.*,

J Biol Chem 263, 9470~9475, 1988]. However, nowadays binding domains and toxic domains themselves are modified to be made as derivatives for better activity[Reference: (Pastan, I., *et al.*, *Science* 254, 1173~1177, 1991)(Pastan, I., *et al.*, *Proc Natl Acad Sci* USA 88, 3358~3362, 1991)(Vitetta, E.S., *et al.*, *Cell Biology* 2, 47~58, 1991)(Allured,V.S., *et al.*, *Proc. Natl. Acad. Sci.* USA 83, 1320~1324, 1986)(Hwang ,J et al., *Cell* 48, 129~136, 1987)].

The modified antibody binding region produced by genetic recombination used for the fusion protein can be classified in 4 types. These are scFv(single chain Fv form) characterized in connecting the minimum binding unit of antibody V_H and V_L with 15 amino acid polypeptide linker(Gly_4Ser_4)[Reference: Buchner, J., *et al.*, *Anal Biochem* 205, 263~70, 1992], dsFv(disulfide-stabilized Fv form) characterized by connecting V_H and V_L via disulfide bonds, pFv(permutated Fv form) characterized in connecting V_H and V_L with base loop and Fab form~~-ete~~. scFv-toxin functional group form has the smallest molecular weight from antibody binding domain produced and for this it was expected to have good penetration ability into cancer tissues showing good cytotoxicity. However, the low productions yield[Reference: (Buchner, J., *et al.*, *Anal Biochem* 205, 263~70, 1992)(Brinkmann. U., *et al.*, *Proc. Natl. Acad. Sci.* USA 88, 8616~8620, 1991)] and short half life in animal blood circulation[Reference: Brinkmann. U., *et al.*, *Proc. Natl. Acad. Sci.* USA 89, 3065~3069, 1992] were problems and there were no effects observed from results of clinical demonstration.

The dsFv-toxin functional group has a similar size to the scFv-toxin functional group and was designed to have a high circulation stability in animal blood. This type of antibody-toxin functional group was more stable than scFv-toxin in blood circulation but in cytotoxicity tests with cultured cells *in vitro,* it had a similar activity to scFv-toxin[Reference: (Pastan, I., *et al., Science* 254, 1173~1177, 1991)(Pastan, I., *et al.*,*Cancer Research* 51, 3781~3787, 1991)]. The

results of dsFv-toxin distribution tests in animals with a radionuclide-labeled dsFv-toxin functional group[Reference: Choi, C.,*et al.*, *Cancer Res.* 55, 5323~9, 1995] showed that dsFv-toxin disappeared from the blood circulation through excretion faster than it could bind and accumulate on cancer cells ~~and accumulate~~.

pFv is made by connecting the β-strand between 3 and 3b on V_L and β-strand between 3 and 3b on V_H[Reference: Brinkmann, U., *et al.*,*J Mol Biol* 268, 107~17, 1997]. However, this form of antibody-toxin functional group showed short half-life ~~rate~~, low production yield , and no improved cytotoxicity effect.

Recombinant Fab-toxin functional group fusion was made to overcome the problem mentioned above[Reference (Ghetie, M.A., *et al.*, 1991)(Kreitman, R.J., *et al.*, *Cancer Res* 53, 819~25, 1993)(Choe, M., *et al.*, *Cancer Res* 54,3406~7, 1994)(Kreitman, R.J *et al., Int J Cancer* 57,856~64, 1994)]. This molecule showed similar half life of activity in blood circulation as the incipient antibody-toxin functional group chemical fusion(mAb-toxin) although it was a recombinant antibody-toxin functional group fusion and was more stable than scFv-toxin functional group, dsFv toxin functional group, pFv-toxin functional group in structure[Reference: (Choe, M., *et al.*, *Cancer Res.* 54, 3460~7, 1994)(Kreitman, R.J., *et al.*, *Int J Cancer* 57, 856~64, 1994)]. Also, the production yield of refolding was 10 times higher in maximum[Reference: (Buchner, J., *et al.*, *Bio/Technology* 9, 157~162, 1991)(Buchner, J., *et al.*, *Anal Biochem* 205, 263~70, 1992)]. Even though the Fab-toxin functional group in blood circulation was more active than scFv, the therapeutic efficacy towards cancer cells in animal model was good or had no big difference according to antibody type. The reason it had no big difference was assumed to be that Fab-toxin functional group has a bigger binding domain that each of the quaternary structure of Fab didn't form properly during refolding or it has two more intrachain disulfide bond to make disulfide bond incomplete or make disulfide

bond scrambled or the cysteins for interchain disulfide bond between heavy and light chain did not form disulfide bond completely or got scrambled.. Therefore, the complete structure formation of molecule was disturbed to have abnormal binding affinity against antigen and these may have mixed with the normal molecules to lower the efficacy[Reference: Choe, M., *et al.*, *Cancer Res* 54. 3460~7, 1994].

In this way, the Fab-toxin functional group has weak binding affinity ~~because~~ as the isolation and removal of the inactivated molecules ~~and molecules~~ with scrambled disulfide bond ~~scrambled~~ or incomplete formed structure is very difficult. It may be possible to remove these inactive molecules in future.

According to these results, the recombinant antibody-toxin functional groups with the best stability in structure and appropriate half-life are Fab-toxin functional group form. ~~To explain this~~The biochemical explanation can be as followings. The~~ly;~~ C_{H1} and C_L and the disulfide bond between light and heavy chain give high stability to quaternary structure, ~~and~~ so the resistance against degradation and clearance will be strong.

The most important issue of the antibody-toxin functional group is stability and affinity.

Therefore, development of new antibody-toxin functional group is needed with improved stability and affinity to have high efficacy for targets and also with high productivity. To increase structural stability, the number of disulfide bond can be increased, but if wrong bonds form or scramblings happen, molecules with inactive structures are produced and the production yield lowers or ~~doesn't produce it at all~~abolishes.

In addition to the described molecules mentioned above, there are toxin functional group-antibody fusions, which have toxin functional group on the amino terminal and antibody binding domain on the carboxyl terminal and they

showed similar results.

Also, antibody derivatives with multiple binding domains were produced to increase binding affinity and they are diabody, triabody, tetrabody, double headed antibody and others[Reference: Takemura S., *et al.*, *Cancer Immunol Immunother.* 51(1): 33~44, 2002]. With iIncreasing the numbers of binding domains two, three, four times and with the affinities between the chains composing binding domains, multiple binding domain derivatives are made. They showed that the binding affinity increased by the increase of binding domain numbers. Tand there was no report before about these molecules being fused with heterogeneous functional groups to produce chimeric fusion. It is a difficult matter to connect binding domain to functional group without hindrance to each other and in forming dimeric form of the chimeric fusion between the functional group themselves. M in forming dimeric form of the chimeric fusion and manufacturers concerned will know that this is the key to successful production of chimeric proteins and their dimers. If the extension chain, which connects binding domain and functional group, disturbs the refolding of the big chains of binding domain or functional group or some hindrance occurs between the big chains, no production will occur.

Also, when the uncoupled Cystecysteine lacking intrachain disulfide bond counterpart cysteine is added in the extension sequence connecting binding domain and functional group, it this may form disulfide bond with other cCysteine, which that has its right counterpart cystein, and this may lead to disulfide bond structure scrambling. In this case the uncoupled Cystecysteine that has no natural disulfide bond counterpart will form wrong disulfide bonds to ruin sterical structure of the molecule and lose its activity. And also, it is natural to think that if the extension chain containing uncoupled Cystecysteine has lots of flexible amino acids to be long making the extension chain have no regular structure, it is easier

for the uncoupled ~~Cystecysteine~~ to intermix with other naturally coupled ~~Cystecysteine~~ in neighboring big structure. The inventors experienced these kinds of failures in producing active dimer molecules and it will be thought as a special and incidental case when the manufacturers concerned produce one of these types of molecule.

If the problems and limits mentioned above are overcome by new findings about forming dimers with disulfide bond generally, the dimer of binding domain(B)- functional group(F) fusion by disulfide bond will be possible by the manufacturers concerned.

On the other side, there were reports about compared studies of various structures of molecules[Reference: Bera, T.K., *et al.*, *Bioconjug Chem* 9(6), 736~4, 1998] but no reports were found about producing dimer[antibody-toxin functional group]$_2$ by using disulfide bonding in a wide range of peptide sequence between antibody- and toxin functional groups fusion to form molecules with double binding valency. Therefore, it has been expected that the manufacturers will be able to know how extension peptide chain to be put in between big sequences of antibody and toxin functional group and how the uncoupled ~~Cystecysteine~~ to be put in to extension peptide chain to form disulfide bonds for dimerization after the understanding about the refolding process forming the tertiary and quaternary structure of the protein.

Brief description of the drawings

Figure 1 is a structure of the dimer of chimeric recombinant binding domain(B)-functional group(F) fusion with double binding valency produced from pMH21, 22, 23 and pMHS22.

Figure 2 shows the construction procedure of pMH21, 22, 23, pMHS22 by PCR.

Figure 3 shows the plasmid map of pMH21, 22, 23, pMHS22.

Figure 4 is a graph showing the production yield according to the position of ~~Cystec~~ysteine at position 1, 4 and 15 on extension peptide chain.

Figure 5 shows antibody-toxin finally purified.

Figure 6 shows the structure of [B3(Fab)-Ext(15CL14FA13)-PE38)$_2$ produced from pCW1.

Figure 7 shows the result profile of Superdex 200 column chromatography and SDS-PAGE analysis.

Figure 8 is a graph showing representative cytotoxicity assay results of [B3 (Fab)-Ext-PE38]$_2$ and control molecule scFv-PE40.

Figure 9 shows the structure of [B3(FabH1)-PE38]$_2$ (=[B3(Fab)-Ext(1CL13FA7)-PE38]$_2$)

Figure 10 shows the TSK-GEL G3000SW column chromatography result of B3 (FabH1)-PE38 and [B3 (FabH1)-PE38]$_2$.

Figure 11 shows the structure of BMH and BM[PEO]$_4$.

Figure 12 shows the structure of antibody-toxin fusion produced from pLSC52, 32, 22.

Figure 13 shows the construction procedure of pLSC52 by PCR.

Figure 14 shows the construction procedure of pLSC32 by PCR.

Figure 15 shows the profile of Source-Q column chromatography and SDS-PAGE results of [B3(Fab)-h(H123-CH3)-PE38R]$_2$.

Figure 16 shows the profile of Superdex 200 column chromatography and SDS-PAGE results of [B3(Fab)-h(H124-Fc)-PE38R]$_2$.

Figure 17 shows the profile of Superdex 200 column chromatography and SDS-PAGE results of [B3(Fab)-h(H124-CH3)-PE38R]$_2$.

Figure 18 shows the profile of Superdex 200 column chromatography and SDS-PAGE results of [B3(Fab)-h(H124-CH2)-PE38R]$_2$.

Disclosure

Technical Problem

The technical task of this invention is fusing binding domain(B) with heterogeneous functional group(F) to form a monomer of chimeric recombinant binding domain-functional group fusion(B-F fusion) and connecting two of these with covalent disulfide-bond-bridge to make a dimer of chimeric recombinant binding domain-functional group fusion([B-F fusion]$_2$) with double binding valency of the monomer.

Technical Solution

This invention is related to a method producing dimer of chimeric recombinant binding domain-heterogeneous functional group fusion([B-F fusion]$_2$) with double binding valency of the monomer by using covalent disulfide-bond-bridge connecting the two ~~of a~~ monomers of chimeric recombinant binding domain- heterogeneous functional group fusion(B-F fusion).

In a concrete way, two monomers ~~is~~ are connected to become a dimer by disulfide-bond-bridge formed by the oxidation reaction between the two uncoupled Cys that is on any of 1~45 amino acid position of extension peptide chain (Ext) which extends from binding domain to functional group for fusion.

At this time, said extension peptide chain(Ext, extension amino acid sequence) is firstly composed of peptide linker(L) from the last of the uncoupled Cys to functional group(end of Ext) and composed of 1~50 amino acids from the last of the uncoupled Cys to functional group. Secondly, said extension peptide chain(Ext, extension amino acid sequence) is composed of peptide linker(L) from the last of the uncoupled Cys to functional group(end of Ext), and said peptide linker(L) is a peptide linker containing an affinity domain(LAD) which has

homomeric self affinity or heteromeric affinity making the domain to assemble and leading to the formation of homomeric multimer or heteromeric multimer, and the amino acid sequence from the end of said affinity domain(AD) to functional group(end of Ext) is composed of 1~50 amino acids. Thirdly, said extension peptide chain(Ext, extension amino acid sequence) is composed of peptide linker(L) from the last of the uncoupled Cys to functional group(end of Ext), and said peptide linker(L) is a flexible amino acid sequence peptide linker(LFA) which contains non-bulky amino acids like Glycine(G) or Alanine(A) or Serine(S) or Glutamine(Q) or Glutamic acid(E) or Asparagine(N), Aspartic acid(D), and the amino acid sequence from the last of the uncoupled Cys to functional group is composed of 1~50 amino acids. Fourthly, said extension peptide chain(Ext, extension amino acid sequence) is composed of peptide linker(L) from the last of the uncoupled Cys to functional group (end of Ext), and said peptide linker(L) is a peptide linker containing an affinity domain(LAD) which has homomeric self affinity or heteromeric affinity making the domain to assemble and leading to the formation of homomeric multimer or heteromeric multimer, and said peptide linker(L) is also a flexible amino acid sequence peptide linker(LFA) which contains non-bulky amino acids like Glycine(G) or Alanine(A) or Serine(S) or Glutamine(Q) or Glutamic acid(E) or Asparagine(N), Aspartic acid(D), and the amino acid sequence from the end of said affinity domain(AD) to functional group(end of Ext) is composed of 1~50 amino acids.

On the other side, the binding domain on the chimeric recombinant binding domain-heterogeneous functional group fusion is composed of multiple chains(B1,B2,...,Bn) and one of the chain(B1) of binding domain is connected to extension peptide chain that has the cysteine for the disulfide–bond-bridge between two monomer and another chain(B2) is connected to heterogeneous functional group.

At this time, said extension peptide chain(Ext) which is connected to one of the chain(B1) comprises firstly, uncoupled Cys on any of 1~45 amino acids position on the extension peptide chain. Secondly said extension peptide chain comprises uncoupled Cys on any of 1~45 amino acids position and comprises peptide linker(L) from the last uncoupled Cys to the end of extension peptide chain. Thirdly, said extension peptide chain comprises uncoupled Cys on any of 1~45 amino acids position and comprises peptide linker(L) from the last uncoupled Cys to the end of extension peptide chain and said peptide linker(L) has homomeric self affinity or heteromeric affinity domain(AD) making the domain to assemble and leading to the formation of homomeric multimer or heteromeric multimer. Fourthly, said extension peptide chain comprises uncoupled Cys on any of 1~45 amino acids position and comprises peptide linker(L) from the last uncoupled Cys to the end of extension peptide chain and said peptide linker(L) has homomeric self affinity or heteromeric affinity domain(AD) making the domain to assemble and leading to the formation of homomeric multimer or heteromeric multimer and said peptide linker(L) comprises flexible amino acid sequence with non-bulky amino acids like Glycine(G) or Alanine(A) or Serine(S) or Glutamine(Q) or Glutamic acid(E) or Asparagine(N), Aspartic acid(D).

At this time, another chain(B2) firstly, has functional group(F) at the end of the chain. Secondly, has functional group(F) at the end of the peptide linker(L) connected to the end of the chain and said peptide linker comprises 1~50 amino acids. Thirdly, has functional group(F) at the end of the peptide linker(L) connected to the end of the chain and said peptide linker(L) is flexible amino acid sequence peptide linker(LFA) which comprises Glycine(G) or Alanine(A) or Serine(S) or Glutamine(Q) or Glutamic acid(E) or Asparagine(N), Aspartic acid(D) and said peptide linker(L) comprises 1~50 amino acids.

Binding domain(B) is adhesive protein or amino acid sequence having

adhesive fragment for instance, antibody, fragment of antibody like scFv, pFv, dsFv, Fab, L(using 1 light chain), LL(using 2 light chains), H(using 1 heavy chain), HH(using 2 heavy chains), diabody, triabody, tetrabody, double-headed antibody, ligands for example, TGF alpha, TGF beta, IL2, IL6, TNF, GMSCF or some fragments having ligand's affinity, all kinds of ligand receptors for example, insulin receptor, TBP1, TBP2, IFN alpha or beta receptor, gonadotropin receptor or some fragments having receptor's affinity and sequences having binding affinity.

Functional group(F) is a functional group with all kinds of physiological functions including enzymes used in prodrug transformation, detection, decomposition, formation of materials and proteins containing toxin-functional group which has cytotoxicity, organisms like viruses for gene therapy, compounds with cationic tail for delivering DNA, drug compounds, liposome for drug delivery, biosensor for detecting real time target molecule.

The dimer of chimeric recombinant binding domain(B)-heterogeneous functional group(F) fusion was the first to be formed by using disulfide-bond-bridge to connect monomers having twice as high binding valency, and it has higher functional efficiency to its targets and the production yield is high containing said extension peptide chain.

Namely, from one point of view, this invention offers dimer of chimeric recombinant binding domain-functional group fusion($[B\text{-}F\ fusion]_2$) with double binding valency by using disulfide-bond-bridge connecting the two of a monomer of chimeric recombinant binding domain-functional group fusion([B-F fusion]).

In another point of view, this invention offers polypeptides used in producing dimer of chimeric recombinant binding domain-functional group fusion($[B\text{-}F\ fusion]_2$) with double binding valency by using disulfide-bond-bridge connecting the two of a monomer of chimeric recombinant binding domain-

functional group fusion([B-F fusion]).

In another point of view, this invention offers recombinant plasmid containing genes coding polypeptides used in producing dimer of chimeric recombinant binding domain-functional group fusion($[B\text{-}F\ fusion]_2$) with double binding valency by using disulfide-bond-bridge connecting the two of a monomer of chimeric recombinant binding domain-functional group fusion([B-F fusion])..

Something to make clear in this invention is concluding not only the amino acid sequence of binding domain(B)-functional group(F) protein but concluding all the nucleotide sequences coding the same protein. Actually, all the nucleotide sequences means all the different DNA sequences which codes the same amino acid using different codons to code same amino acids.

PCR is mostly used to produce chimeric recombinant binding domain(B)-functional domain(F) fusion coding sequences by using oligonucleotide primers made from clones which has genes coding the protein we want.

The invention chimeric recombinant binding domain(B)-functional domain(F) fusion can be expressed in high class eukaryotic cell(for example, yeast, insects or mammalian cells) or prokaryotic cell of microorganism using appropriate expression vector. Any method known in this field can be used.

For example, chimeric recombinant binding domain(B)-functional domain(F) fusion protein coding DNA produced from said any method referred above can be inserted into appropriate expression vector. Double strand cDNA is inserted into the vector by using synthesized DNA linker or blunt-ended DNA ligation or restriction enzyme treated DNA ligation or terminal polymerase treated DNA ligation. DNA ligase is used in DNA molecules ligation and alkaline phosphatase is used in removing the phosphate group.

Expression vector must have specific nucleotides elements for mRNA transcription and translation into protein from the coding DNA sequence.

Promoter is needed for the RNA polymerase to recognize for transcription. RNA polymerase binds to this promoter to start transcription. Promoters used are various on general but has different efficiency.

In another point of view, this invention offers host cells including recombinant vectors cloned with genes coding polypeptides used in producing dimer of chimeric recombinant binding domain-functional group fusion([B-F fusion]$_2$) by using disulfide-bond-bridge connecting the two of a monomer of chimeric recombinant binding domain-functional group fusion([B-F fusion]).

According to eukaryotic hosts, it varies with control sequences for transcription and translation. These are regulatory signals related to highly expressed genes and can be gained from viruses like adenovirus, cow papillomavirus, anthropoid virus. For example, there are TK promoter from Herpes virus, SV40 early promoter, gal4 gene promoter from yeast and more. There are transcription start control signals that can both inhibit or promote the expression of the gene to be selected for use.

Transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection and others can insert the expression vector into host cell.

Host cells are prokaryotic or eukaryotic cells. Eukaryotic cells are preferable for example, mammalian cells like human, monkey, mouse, Chinese hamster ovarian cell(CHO) because these cells offer correct folding or modification of proteins like glycosylation. Also, in yeast cells modification including glycosylation takes place after protein expression. Yeast cells provide high copy numbers of recombinant vectors in the cell and transcription efficiency is high. The yeast recognizes the guidance sequence of protein secretion signal of the cloned mammalian gene and secretes the peptide(precursor protein) which has the signal.

In another point of view, this invention offers method to produce chimeric recombinant binding domain-functional group fusion($[B\text{-}F\ fusion]_2$) through host cells including recombinant vectors cloned with genes coding polypeptides used in producing dimer of chimeric recombinant binding domain-functional group fusion($[B\text{-}F\ fusion]_2$) by using disulfide-bond-bridge connecting the two of a monomer of chimeric recombinant binding domain-functional group fusion([B-F fusion]).

In another point of view, this invention offers pharmaceutical compounds containing chimeric recombinant binding domain-functional group fusion($[B\text{-}F\ fusion]_2$).

The dimeric form of chimeric recombinant binding domain-functional group fusion($[B\text{-}F\ fusion]_2$) with double binding valency is indicated as a formula below.

$[Binding\ domain(B)\text{-}\ Extension\ peptide\ chain(Ext)\text{-}Funtional\ group(F)]_2$ ....(I)

The binding sequence(B) in said formula(I) above is adhesive protein or adhesive fragment containing amino acid sequence ;

Ext is extension peptide chain(extension sequence) extended from binding domain(B) to functional group(F) to fuse them, and disulfide-bond-bridge is formed between the monomers by the oxidation of two uncoupled Cys on the extension peptide chain(Ext) to produce dimer, or said extension peptide chain comprises peptide linker(L) from the last uncoupled Cys to the end of extension peptide chain or said peptide linker is an affinity domain(AD) containing peptide linker(LAD) which has homomeric self affinity or heteromeric affinity leading to the formation of homomeric multimer or heteromeric multimer domain, or said peptide linker(L) is flexible amino acid(FA) sequence peptide linker(LFA) which

has non-bulky amino acids like Glycine(G) or Alanine(A) or Serine(S) or Glutamine(Q) or Glutamic acid(E) or Asparagine(N), Aspartic acid(D) or said peptide linker having affinity domain(AD) with homomeric self affinity or heteromeric affinity comprising muldimer domains and having non-bulky amino acids like Glycine(G) or Alanine(A) or Serine(S) or Glutamine(Q) or Glutamic acid(E) or Asparagine(N), Aspartic acid(D) to be a flexible amino acid sequence peptide linker with affinity domain(LADFA) so to be a sequence as [B-Ext or Ext(L) or Ext(LAD) or Ext(LFA) or Ext(LADFA)-F] in its monomer form ;

F is for physiological functional group like enzyme, protein with physiological (e.g. toxic) function, organisms like viruses, compounds, drug compounds for treatment, liposome, biosensor, pro-drug and more[Reference: (Farah, R.A.,*et al.*,*Crit. Rev. Eukaryot. Gene Expr.*8, 321~356, 1998) (Trail, P.A., *et al.*, *Science* 261, 212~215, 1993)(Hinman, L.M., *et al.*, *Cancer res.* 53, 3336~3342, 1993)(Pastan, *I. Biochem. Biophys. Acta* 1333, C1~C6, 1997)(Kreitman, P.J., *et al.*,*J. Clin. Oncol.* 18, 1622~1636, 2000)(Zalutsky, M.R & Vaidyanathan, G. Curr.Pharm.Des. 6, 1433~1455, 2000)(Goldenburg, D.M. in Clinical Uses of Antibodies (eds Baum,R.P.,*et al.*)1~13(Kluwer academic, The Netherlands, 1991)(Lode,H.N.& Reisfield, R.A *Immunol.Res.* 21, 279~288, 2000) (Penichet,M.L & Morrison, S.L. *J.Immunol.Methods* 248, 91~101, 2001)(Lasic, D.D & Papahadjopoulos, D. *Science* 267, 1275~1276, 1995) (Park.J.W., *et al., Proc. Natl Acad. Sci.* USA 92, 1327~1331)(Niculescu-Davaz, I., *et al.*, *Anticancer Drug Des.* 14, 517~538, 1999)(SToldt,H.S., *et al.*, *Eur. J. Cancer* 33, 186~192, 1997))]

In another mode, the dimeric form of chimeric recombinant binding domain-functional group fusion([B-F fusion]$_2$) with double binding valency is indicated as a formula below.

[Antibody binding domain-Ext- F]$_2$....(II)

The antibody binding domain in formula(II) is antibody or amino acid sequence comprising adhesive fragment of antibody.

Ext is extension peptide chain(extension sequence) extended from binding domain(B) to functional group(F) to fuse them, and disulfide-bond-bridge is formed between the monomers by the oxidation of two uncoupled Cys on the extension peptide chain(Ext) to produce dimer, or said extension peptide chain comprises peptide linker(L) from the last uncoupled Cys to the end of extension peptide chain or said peptide linker is an affinity domain(AD) containing peptide linker(LAD) which has homomeric self affinity or heteromeric affinity leading to the formation of homomeric multimer or heteromeric multimer domain, or said peptide linker(L) is flexible amino acid(FA) sequence peptide linker(LFA) which has non-bulky amino acids like Glycine(G) or Alanine(A) or Serine(S) or Glutamine(Q) or Glutamic acid(E) or Asparagine(N), Aspartic acid(D) or said peptide linker having affinity domain(AD) with homomeric self affinity or heteromeric affinity comprising multimer domains and having non-bulky amino acids like Glycine(G) or Alanine(A) or Serine(S) or Glutamine(Q) or Glutamic acid(E) or Asparagine(N), Aspartic acid(D) to be a flexible amino acid sequence peptide linker with affinity domain(LADFA) so to be a sequence as [B-Ext or Ext(L) or Ext(LAD) or Ext(LFA) or Ext(LADFA)-F] in its monomer form ;

F is for physiological functional group like enzyme, protein with physiological (e.g. toxic) function, organisms like viruses, compounds, drug compounds for treatment, liposome, biosensor, pro-drug and more.

In another mode, the dimeric form of chimeric recombinant binding domain-functional group fusion($[B\text{-}F\ fusion]_2$) with double binding valency is indicated as a formula below

$[Fab\text{-}Ext\text{-}F]_2$....(III)

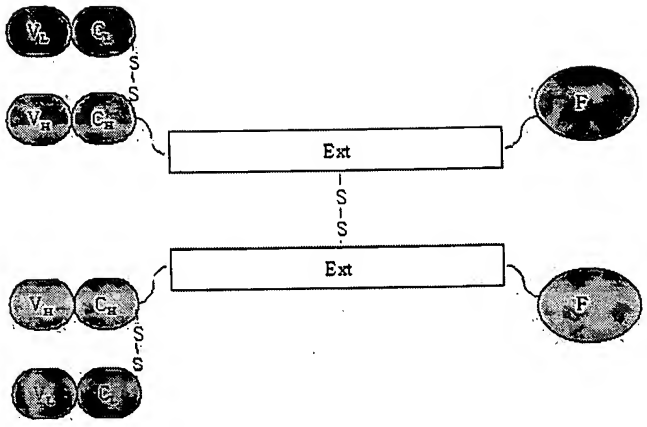
Fab in the said formula(III) is Fab fragment of antibody.

Ext is extension peptide chain (extension sequence) extended from binding domain(B) to functional group(F) to fuse them, and disulfide-bond-bridge is formed between the monomers by the oxidation of two uncoupled Cys on the extension peptide chain(Ext) to produce dimer, or said extension peptide chain comprises peptide linker(L) from the last uncoupled Cys to the end of extension peptide chain or said peptide linker is an affinity domain(AD) containing peptide linker(LAD) which has homomeric self affinity or heteromeric affinity leading to the formation of homomeric multimer or heteromeric multimer domain, or said peptide linker(L) is flexible amino acid (FA) sequence peptide linker(LFA) which has non-bulky amino acids like Glycine(G) or Alanine(A) or Serine(S) or Glutamine(Q) or Glutamic acid(E) or Asparagine(N), Aspartic acid(D) or said peptide linker having affinity domain(AD) with homomeric self affinity or heteromeric affinity comprising multimer domains and having non-bulky amino acids like Glycine(G) or Alanine(A) or Serine(S) or Glutamine(Q) or Glutamic acid(E) or Asparagine(N), Aspartic acid(D) to be a flexible amino acid sequence peptide linker with affinity domain(LADFA) so to be a sequence as [B-Ext or Ext(L) or Ext(LAD) or Ext(LFA) or Ext(LADFA)-F] in its monomer form ;

F is for physiological functional group like enzyme, protein with physiological(e.g. toxic) function, organisms like viruses, compounds, drug compounds for treatment, liposome, biosensor, pro-drug and more.

In another mode, the dimeric form of chimeric recombinant binding domain-functional group fusion($[B\text{-}F\ fusion]_2$) with double binding valency is indicated as a formula below

[Reference figure 1]



According to the formula above,

V_L is variable region on light chain of monoclonal antibody;

C_L is constant region on light chain of monoclonal antibody;

V_H is variable region on heavy chain of monoclonal antibody;

C_H is constant region on heavy chain of monoclonal antibody;

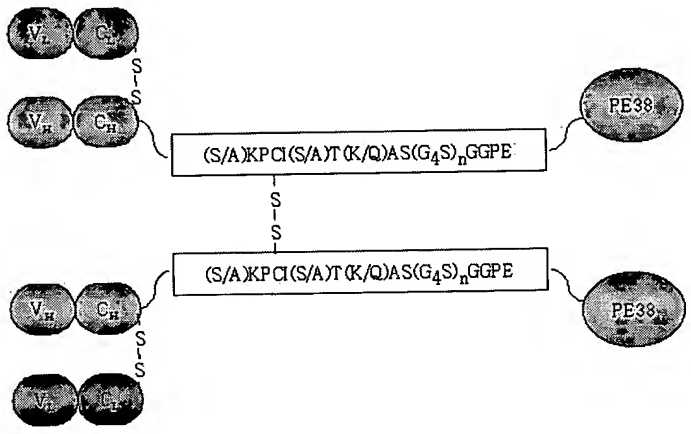
Ext is extension peptide chain(extension sequence) extended from binding domain(B) to functional group(F) to fuse them, and disulfide-bond-bridge is formed between the monomers by the oxidation of two uncoupled Cys on the extension peptide chain(Ext) to produce dimer, or said extension peptide chain comprises peptide linker(L) from the last uncoupled Cys to the end of extension peptide chain or said peptide linker is an affinity domain(AD) containing peptide linker(LAD) which has homomeric self affinity or heteromeric affinity leading to the formation of homomeric multimer or heteromeric multimer domain, or said peptide linker(L) is flexible amino acid (FA) sequence peptide linker(LFA) which has non-bulky amino acids like Glycine(G) or Alanine(A) or Serine(S) or Glutamine(Q) or Glutamic acid(E) or Asparagine(N), Aspartic acid(D) or said peptide linker having affinity domain (AD) with homomeric self affinity or heteromeric affinity comprising multimer domains and having non-bulky amino acids like Glycine(G) or Alanine(A) or Serine(S) or Glutamine(Q) or Glutamic

acid(E) or Asparagine(N), Aspartic acid(D) to be a flexible amino acid sequence peptide linker with affinity domain(LADFA) so to be a sequence as [B-Ext or Ext(L) or Ext(LAD) or Ext(LFA) or Ext(LADFA)-F] in its monomer form ;

F is for physiological functional group like enzyme, protein with physiological(e.g. toxic) function, organisms like viruses, compounds, drug compounds for treatment, liposome, biosensor, pro-drug and more.

More specifically, the dimeric form of chimeric recombinant binding domain-functional group fusion([B-F fusion]$_2$) with double binding valency is indicated as a formula below

[Reference figure 2]



According to the formula above,

V_L is variable region on light chain of monoclonal antibody;

C_L is constant region on light chain of monoclonal antibody;

V_H is variable region on heavy chain of monoclonal antibody;

C_H is constant region on heavy chain of monoclonal antibody;

A is Alanine;

C is ~~Cystecysteine~~;

E is Glutamic acid;

G is Glycine;

I is Isoleucine;

K is Lysine;

P is Proline;

Q is Glutamine;

S is Serine;

T is Threonine;

n is 1 or 2 or 3;

PE38 is 38kDa exotoxin derivative of *Pseudomonas aeruginosa* which has been truncated of amino acid sequences of domain Ia and II that are not required for cytotoxicity.

The inventors used the previous recombinant binding domain(B)-functional domain(F) fusion model to invent [B3(Fab)-(S/A)KPCI(S/A)T(K/Q)AS(G_4S)nGGPE – toxin-functional group]$_2$ having twice the adhesion valency and using monoclonal antibody B3 as a model binding domain. Antibody-toxin functional group fusion made in the meantime was monovalent but the invented dimeric antibody-toxin functional group fusion is divalent so it will display much more cytotoxicity towards cultured cancer cells. This molecule is produced by modifying Fd-CKPSISTKASGGPE-toxin functional group chain among Fd-CKPSISTKASGGPE-toxin functional group chain and L chain which are used in composing monovalent Fab-CKPSISTKASGGPE-toxin functional group formed monomer. The modified chain was made by changing the position of uncoupled ~~Cystec~~ysteine with Serine coming after ~~Cystec~~ysteine. (S/A) means Serine or Alanine and (K/Q) means Lysine or Glutamine. (G_4S)n peptide chain is (GGGGS) or (GGGGSGGGGS) or (GGGGSGGGGSGGGGS) and it's in the middle of the KASGGPE to have Fd-(S/A)KPCI(S/A)T(K/Q)AS(G_4S)nGGPE-PE38 structure and mixing and refolding this with L chain makes an activated molecule. The divalent dimer [Fab-

(S/A)KPCI(S/A)T(K/Q)AS(G_4S)nGGPE – PE38]$_2$ is formed by disulfide-bond-bridge connecting two Fab-(S/A)KPCI(S/A)T(K/Q)AS(G_4S)nGGPE – PE38 by the fourth amino acid , uncoupled ~~Cystec~~ysteine. This molecule keeps the stability of the Fab-toxin functional group structure and targeting efficiency too.

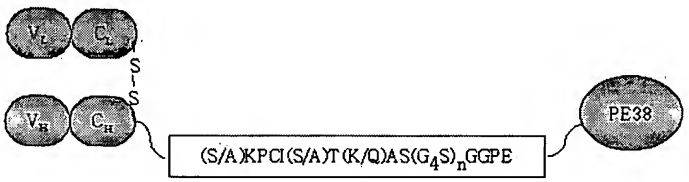
The invention dimer of chimeric recombinant binding domain-functional group fusion([B-F fusion]$_2$) by using disulfide-bond-bridge connecting the two of a monomer of chimeric recombinant binding domain-functional group fusion(B-F fusion) has extension peptide chain(extension amino acid sequence, Ext) extended from binding domain(B) to functional group(F) to fuse them, and disulfide-bond-bridge is formed between the monomers by two uncoupled oxidized Cys on the extension peptide chain(Ext) to produce dimer, or said extension peptide chain comprises peptide linker(L) from the last uncoupled Cys to the end of extension peptide chain or said peptide linker is an affinity domain containing peptide linker(LAD) which has homomeric self affinity or heteromeric affinity comprising homomeric multimer or heteromeric multimer domain, affinity domain(AD) or said peptide linker(L) is flexible amino acid (FA) sequence peptide linker(LFA) which has non-bulky amino acids like Glycine(G) or Alanine(A) or Serine(S) or Glutamine(Q) or Glutamic acid(E) or Asparagine(N), Aspartic acid(D) or said peptide linker having affinity domain (AD) with homomeric self affinity or heteromeric affinity comprising multimer domains and having non-bulky amino acids like Glycine(G) or Alanine(A) or Serine(S) or Glutamine(Q) or Glutamic acid(E) or Asparagine(N), Aspartic acid(D) to be a flexible amino acid sequece peptide linker with affinity domain (LADFA) so to be a sequence as [B-Ext or Ext(L) or Ext(LAD) or Ext(LFA) or Ext(LADFA)-F] in its monomer form. And the dimer has decreased three-dimensional hindrance between the two functional groups while being produced and they are produced through mixing and refolding the polypeptide obtained

from the host cells containing recombinant plasmid with the Ext sequences for dimerization.

In the following, the double binding valency dimer of chimeric recombinant binding domain(B)-functional domain(F) fusion will be described in details and through this description the manufacturers concerned will be able to produce any related dimer of chimeric recombinant binding domain(B)-functional domain(F) fusion.

In another mode, this invention offers polypeptides for producing dimeric chimeric recombinant binding domain-functional group fusion([B-F fusion]$_2$) and it is indicated as a formula below.

[Reference figure 3]



In another mode, this invention offers recombinant plasmid that expresses said polypeptide.

In another mode, this invention offers methods to produce said divalent recombinant antibody-toxin functional group fusion by culturing host cells with recombinant plasmid expressing said polypeptide gene and other host cells with plasmid expressing light chain which includes V_L and C_L and joining them together and refolding them.

The divalent recombinant antibody-toxin functional group fusion specifically binding its antibody to cancer cell and connecting the toxin functional group is anti-cancer therapeutic agent and it kills the cancer cells without damaging other normal cells.

B3(Fab)-PE38 is produced from the fusion of Fab of monoclonal

antibody B3 and PE38 which is truncated type of *Pseudomonas aeruginosa* exotoxin and it doesn't have uncoupled ~~Cyste~~cysteine which means it can only be a monomer. The [B3(Fab)-Ext-PE38]$_2$ which belongs to divalent chimeric recombinant binding domain(B)-functional domain(F) fusion is derived from B3(Fab)-PE38 and the Fab of antibody is connected to functional group with (S/A)KPCI(S/A)T(K/Q)AS(G_4S)nGGPE.

The monoclonal antibody B3 used in this invention binds directly to LeY type carbohydrate antigen found not only in mucinous cancer like colon carcinoma, stomach cancer, ovarian cancer, breast cancer and lung cancer but also various epidermoid carcinomas.

Pseudomonas aeruginosa exotoxin PE –derived PE38 was used as toxin functional group in this invention. PE is composed of three structural domains. Domain 1 at the amino terminal binds with the cell, domain 2 enables the transport of the protein into the cell, domain 3 is at the carboxy terminal having cytotoxic enzymatic activity. Truncating needless amino acids not used for cytotoxic enzymatic activity from domain 1, 2 produced the 38kDa PE38.

The B3 monoclonal antibody is dimer form in nature as [Fab-Fc]$_2$ because of the three ~~Cyste~~cysteine in the hinge. The inventors used 1 uncoupled ~~Cyste~~cysteine, or 3 uncoupled ~~Cyste~~cysteines although 3 uncoupled ~~Cyste~~cysteines have much higher risk of mixing disulfide bonding while refolding and showed that using multiple numbers of uncoupled ~~Cyste~~cysteine can also produce physiologically active dimers. Manufacturers concerned know the dimer produced with multiple disulfide bonds using multiple uncoupled ~~Cyste~~cysteins will be highly thermodynamically stable.

The extension peptide chain(Ext) (S/A)KPCI(S/A)T(K/Q)AS(G_4S)nGGPE has a flexible peptide linker(LFA) I(S/A)T(K/Q) AS(G_4S)nGGPE including GASQEND. Also the thiol group of the

uncoupled ~~Cystecysteine~~ on Ext enables disulfide bonding to form divalent chimeric recombinant antibody-toxin functional group fusion[B3(Fab)-Ext-PE38]$_2$ from two polypeptide[B3(Fab)-Ext-PE38].

The peptide linker following the uncoupled ~~Cystecysteine~~ in the extension peptide chain reduces the three-dimensional hindrance between the two toxin functional group PE38 helping dimerization. Also under same condition, when the number n increases from 1 to 3, the production yield increased. This means while the dimer has 50kDa Fab sequence and 38kDa PE38, until the number of GASQEND which is in flexible peptide linker(LFA) increases to 21, the dimerization will increase.

The inventors realized that dimer is not only formed by specific numbers and specific location of the uncoupled ~~Cystecysteine~~ and specific linker(L) amino acid sequence that they obtained by chance but, multiple numbers and certain range of locations of Cys, certain range of amino acid sequence of linker(L) can make dimers too. The manufacturers concerned can easily predict that if the size of the binding domain(B) differs and the functional domain(F) also differs, the trend will differ too. So to speak, in case of small sized binding domain and functional domain fusion, dimerization will occur even under peptide linker with only 1 amino acid and if there are too many GASQEND of the flexible peptide chain(LFA) in the extension peptide chain, it will rather disturb refolding of the binding domain and functional domain because of the easy mutual interference and result in decrease of the active molecule yield. Therefore, it is important to find out the range of the position of the uncoupled Cys and the range of the length of the amino acid sequence(the length of L,LFA or the length between AD and F in LAD,LADFA) needed in the peptide linker of extension peptide chain that makes dimerization possible without disulfide bond scrambling and inter-domain refolding interference. Naturally, ~~the big sized binding~~

~~domain(B) and functional domain(F) fusion will need more than 1 flexible amino acid of GASQEND for dimerization and longer length~~ it can be predicted easily among manufacturers concerned that the big sized binding domain(B) and functional domain(F) fusion will need more than 1 flexible amino acid of GASQEND and longer length for dimerization.

The divalent chimeric recombinant antibody-toxin functional group fusion dimer[B3(Fab)-Ext-PE38]$_2$ is produced by ~~applying fusion of~~fusing the antibody gene and toxin functional group gene together and expressing it in *E.coli.*(Reference:Allured *et al.*,1986; Brinkmann *et al.*, 1992; Pai *et al.*,1991: Roscoe *et al.*,1997)

In detail, recombinant vector expressing polypeptide B3(Fd)-Ext-PE38 is produced in this invention. The polypeptide B3(Fd)-Ext-PE38 has a polypeptide chain (S/A)KPCI(S/A)T(K/Q)AS(G$_4$S)nGGPE between Fd and PE38. Therefore, in this case, flexible amino acid peptide linker(LFA) of extension peptide chain(Ext) is "I(S/A)T(K/Q)AS(G$_4$S)nGGPE" and the constitution of B3(Fd)-Ext-PE38 is same as B3(VH)-B3(CH1)-(S/A)KPCI(S/A)T(K/Q)AS(G$_4$S)nGGPE-PE38. The uncoupled ~~Cyste~~cysteine on extension peptide chain forms disulfide bond-bridge between two Fab-Ext-PE38. The decrease of three dimensional hindrance between two PE38 by flexible amino acid following uncoupled cysteine on the extension chain ~~enables~~ makes dimerization of B3(Fab)-Ext-PE38 ~~easily by flexible amino acid following uncoupled Cysteine on the extension chain~~.

The extension peptide chain is for amicable, independent refolding without disturbances between antibody and PE38 ~~toxin functional group~~ in producing recombinant protein antibody-toxin functional group fusion.

The Proline(P) on the end of extension peptide chain (S/A)KPCI(S/A)T(K/Q)AS(G$_4$S)nGGPE cuts off the continuation of refolding motifs to prevent each refolding motif from interfering neighbor refolding domain

during the process of refolding of antibody and toxin functional group.

The divalent recombinant antibody-toxin functional group fusion is produced by culturing the host cells having recombinant vectors cloned with B3(Fd)-Ext-PE38 and light chain of B3 respectively to gain polypeptides of these and then mixing these to refold. The cloned gene is expressed and gained as inclusion body in *E. coli.* Polypeptide is refolded and the active products are separated.

The method for refolding can follow well-known procedures and some references describe the methods[Reference: Buchner,J.,*et al.*, *Anal .Biochem.* 205(2), 263~70 (1992), Buchner, J., *et al.*, *Biotechnonlogy*, 9:157~162 (1991)]

Typical ID50 value of divalent recombinant antibody-toxin functional group fusion$[B3(Fab)\text{-}Ext\text{-}PE38]_2$ was measured on A431 adenosquamous carcinoma cell line at 4ng/㎖, CRL1739 stomach cancer cell line at 1ng/㎖, MCF-7 breast cancer cell line at 5ng/㎖. The divalent recombinant antibody-toxin functional group fusion$[B3(Fab)\text{-}Ext\text{-}PE38]_2$ showed nearly 12 times higher cytotoxicity than the monovalent antibody-toxin functional group B3(scFv)-PE40.

This invention dimer of chimeric recombinant binding domain(B)-functional group(F) fusion offers pharmaceutical drug formulation which includes excipients like carriers or diluents. Pharmaceutical drug formulation containing dimer of chimeric recombinant binding domain(B)-functional group(F) fusion can be produced by commonly used method and prescribed in an appropriate way.

Dimer of chimeric recombinant binding domain(B)-functional group(F) fusion can be used as a drug alone or with radiotherapy, chemotherapy(cell growth inhibitor, cytotoxic agents, antibiotic agents, alkylating agents, anti-metabolic agents, hormones, immunological agents, interferon agents, cycloxygenase inhibitor(e.g. COX-2 inhibitor), metalomatrixprotease inhibitor,

telomerase inhibitor, tyrosine kinase inhibitor, anti-growth factor receptor agents, anti-HER agents, anti-EGFR agents, anti-angiogenesis agents, farnesyl transferase inhibitor, ras-raf signal transduction inhibitor, cell cycle inhibitor, cdk inhibitors, tubulin binders, topoisomerase linhibitor, topoisomerase II inhibitor etc.) and others.

For example, dimer of chimeric recombinant binding domain(B)-functional group(F) fusion can be prescribed with liposomal agents containing more than one chemical agents (e.g.taxane, taxane-derivatives, capsulated taxane, CPT-11, camptothesin-derivatives, anthracycline glycoside for example, idarubicin, epirubicin, etoposide, navelbine, vinblastine, carboplatine, cisplatin, estramustine, celecoxib, Sugen SU-5416, Sugen SU-6668, Herceptin etc.) used in chemotherapy.

If the mixed formulation of dimer of chimeric recombinant binding domain(B)-functional group(F) fusion and chemicals is not appropriate, they can be used on sequential order.

Dimer of chimeric recombinant binding domain(B)-functional group(F) fusion is administered through common routes and the dosage is decided according to the age, weight, condition and medication route of the drug. For example, the suitable dosage for dimer of chimeric recombinant binding domain(B)-functional group(F) fusion can be 10 ~2000mg , once to 5 times a day. The dimer of chimeric recombinant binding domain(B)-functional group(F) fusion can be medicated in various forms like oral delivery system as tablets, capsules, sugar-coated tablets, film-coated tablet, solution, suspension or suppositories systems or parenteral methods as injection into muscles, vein and/or central canal and/or spinal cord.

Solid drugs for oral application contain active components with diluents (e.g. lactose, dextrose, sucrose, cellulose, corn starch, potato starch),

lubricants(e.g. silica, talc, stearate, magnesium, calcium stearate and/or polyethylene glycol), bonding agents(e.g. starch, Arabic gum, gelatin methylcellulose, carboxymethyl cellulose, poly(N-vinyl pyrrolidone), disintegrants(e.g. starch, alginate, sodium starch, glycolate), formal mixtures, dyes, sweetenings, wetting agents(e.g. lecithin, polysorbate, lauryl sulfate) and pharmaceutically inactive agents commonly used in pharmaceutics. Using common methods, for example mixing, granulation, tablet formation, sugar or film coating can produce these drugs.

Liquid dispersion for oral application is for example, syrup, emulsion, and suspension. Emulsion and suspension can contain carriers and there are natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose and polyvinyl alcohol, which are used. Suspension or solution for injection into the muscle may contain active components with pharmaceutically allowed carriers like distilled water, olive oil, ethyl oleiate, glycol(e.g. propylene glycol) and appropriate dosage of lidocaine hydrochloride if needed. Carriers used for injections or infusions are distilled water and preferably they are sterilized, hydrogenous, isotonic solution or they may contain propylene glycol as a carrier. Suppositories use carriers like cocoa butter, polyethylene glycol, polyoxyethylene sorbitan, fatty acid ester surface-active agent, lecithin.

The following will describe this invention more minutely in detail using examples but it does not limit the boundary of the claim.

Advantageous Effects

The invention dimer of chimeric recombinant binding domain(B)-functional group(F) fusion formed via disulfide-bond-bridge between monomeric chimeric recombinant binding domain(B)-functional group(F) fusion is valuable for the pharmaco-medical industry since it is the first dimer formed by covalent

disulfide bond to have twice the binding valency and high structural stability and has excellent functional efficiency towards its targets and has high production yield by containing said ~~flexible~~ extension peptide chain ~~(LFA)~~.

The best modes for working the invention

Example 1: The production of dimers having an Ext (4CLFA5X = 4CL15FA11, 4CL20FA16, 4CL25FA21, AQ4CL20FA16) with a fixed uncoupled cysteine residue at the fourth amino acid position and displaying five amino acid increments in their flexible linker sequences (LFA)

[B3(Fab)-Ext(4CLFA5X = 4CL15FA11, 4CL20FA16, 4CL25FA21, AQ4CL20FA16)-toxin]$_2$ has an uncoupled cysteine residue fixed at the 4th position and has an extension peptide chain with five amino acid increments in the flexible amino acid linker(LFA) in its monomeric unit.

In this example, the uncoupled cysteine in the fourth location from the Fab of B3 antibody is fixed to induce dimer formation, while in the extension peptide, different number of flexible amino acids were put in between the Fd and toxin domains so as to compare the fluctuation in the production yields caused by the differences in steric bulk. 11, 16, 21 flexible amino acids which make the length of Linker to be 15, 20, 25 were used on this test. Alanine was used instead of serine for the position of cysteine at the natural antibody hinge sequence. The OH group of two serines takes more three-dimensional space than the natural cystine disulfide bond in dimer form. The reduced number of cysteine residues in the extension sequence derived from the antibody hinge sequence prevents scrambling of disulfide bonds.

The presented four types of [B3(Fab)-Ext(4CLFA5X)-PE38]$_2$ molecules had the same disulfide bonds formed between uncoupled cysteine residues fixed

at the 4th position in Ext of each monomer to make its interference on the formation of three dimensional structure and intermixing of disulfide bonds equal. And the amino acids following the uncoupled ~~Cystecysteines~~ were left the same except that the numbers of flexible amino acids were increased to see the effect of the steric tolerance given by the increased length of the LFA. Adding flexible amino acids led to 12~17 times higher production yield of these molecules than molecules produced before.

Therefore, this example, compared with the accumulated previous results, shows the disulfide bond between uncoupled Cys on Ext of the two fusion monomers of large molecular weight 50kD B3 antibody and 38 kD Fab domain can be formed to produce the dimer on any position within a certain range of the Ext and not at a specific point of Ext without interfering the large neighboring domains. Thus this example shows definitely that the position of the uncoupled cysteine in Ext can be chosen generally within a certain range and the increase in the number of flexible amino acids in the range of 11~21, which in turn makes the length of LFA to be 15~25, helps the disulfide bond formation for dimerization.

(Apparatus and Methods)

E.coli BL21(DE3) was used for protein expression. Cancer cell lines used in cytotoxicity test were A431, CRL1739, MCF7, KB3-1. pMH21, 22, 23, pMHS22 is plasmid coding B3(Fd)-Ext(4CL15FA11, 4CL20FA16, 4CL25FA21, AQ4CL20FA16)-PE38 and plasmid pMC74, pCE2 is used for construction of the plasmids and plasmid which offers the light chain that matches with pMH21, 22, 23, pMHS22 is pMCH75. The description of the plasmid, cell line and primer used in the experiment is on table 1, 2 and 3 respectively.

Table 1

Plasmid Name	Polypeptide Chain Encoded

pMC74	B3(Fd)-SKPSISTKASGGPE-PE38REDLK
pCE2	B3(Fd)-SKPCISTKASGGPE- PE38REDLK
pMCH75	H6-B3(L)
pMH21	B3(Fd)-Ext(4CL15FA11)-PE38 =B3(Fd)-SKPCISTKAS$(G_4S)_1$GGPE- PE38REDLK
pMH22	B3(Fd)-Ext(4CL20FA16)-PE38 =B3(Fd)-SKPCISTKAS$(G_4S)_2$GGPE- PE38REDLK
pMH23	B3(Fd)-Ext(4CL25FA21)-PE38 =B3(Fd)-SKPCISTKAS$(G_4S)_3$GGPE- PE38REDLK
pMHS22	B3(Fd)-Ext(AQ4CL20FA16)-PE38 =B3(Fd)-**A**KPCI**A**T**Q**AS$(G_4S)_2$GGPE- PE38REDLK

Table 2

cell line	cell type	B3 antigen expression	Media Used
A431	Epidermoid	+++	RPMI 1640, 10% FBS
CRL-1739	Gastric	+	RPMI 1640, 10% FBS
MCR-7	Breast adenocarcinoma	+++	RPMI 1640, 5% FBS
KB3-1	Epidermoid cervix	-	DMEM, 5% FBS

Table 3

Name of the primer	Sequence 5'-3'
Primer MH-1	TAA TAC GAC TCA CTA TAG GGA GA
Primer MH-2	AGA TCC GCC ACC ACC AGA AGC TTT TGT ACT TAT GCT
Primer MH-3	CCA GAT CCG CCA CCA CCA CTT CCC CCT CCC CCG GAA GCT TTT GTA CTT ATG CTA GGC TTA CT
Primer MH-4	TGC TGG TGG CGG ATC TGG AGG TCC CGA GGG CGG CAAG C
Primer MH-5	TGG TGG TGG CGG ATC TGG AGG TGG CGG AAG CGG AGG TCC CGA GGG CGG CAG C
Primer MH-6	GCC GCG GGT GCT GAA GCT GAC GTC GCC GCC GTC
Primer MH-7	GGG AAT TCA TTA AGC TTG TGT AGC TAT GCA AGG CTT AGC ACC ACA

(Reagents Used)

Tryptone, yeast extract, agar(Difco co.), minerals(Junsei co.) was used in culture medium for bacterial culture. Ampicilin (Sigma chemical co.)at a final concentration of 200μg/mL was chosen for the bacterial selection and added to the medium.

*Nde*I, *Hind*III, *Sal*used for plasmid construction was products from NEB co. Ex *Taq* polymerase for PCR amplification and T4 DNA ligase for ligation were products from TaKaRa co. Coomassie Plus Protein Assay Reagent and BSA standard protein from Pierce co. were used for protein analysis. Buffers needed

for dialysis, denaturation, refolding, protein purification were from Sigma. Isopropyl-β-thiogalctopyranoside(IPTG) for protein induction and urea for dialysis were products from Duchefa co. Q-sepharose(Pharmacia Biotech) and Source Q(Pharmacia Biotech) were used as column for purification of protein which is anion exchange chromatography and Superdex-200(Pharmacia Biotech) was used for size exclusion chromatography.

(Plasmid construction)

Construction of plasmid expressing B3(Fd)-Ext-PE38 recombinant protein was made by splicing PCR using pMC74(Fd-PE38) as a template to insert $(G_4S)_n$ inside of the KASGGPE in existing Fd-PE38. This $(G_4S)_n$–inserted coding sequence was exchanged with appropriate part of plasmid pCE2(vector which expresses B3(Fd)-SKPCISTKASGGPE-PE38) using *Hind* III, *EcoR*I.

Plasmid pMHS22(B3(Fd)-Ext(AQ4CL20FA16)-PE38) which has exchanged the ~~Cystecysteine~~ on the natural hinge sequence to Alanine was constructed by PCR using pMH22(B3(Fd)-Ext(4CL20FA16)-PE38) as a template to get a fragment and then exchanged with appropriate part of pMH22.

pMH21, 22, 23 which expresses [B3(Fd)-Ext(4CL15FA11, 4CL20FA16, 4CL25FA21)-PE38]$_2$ and pMHS22 which expresses B3(Fd)-Ext(AQ4CL20FA16)-PE28 are shown in figure 1. Figure 2 shows the construction procedure for plasmid.

(Protein expression and isolation of inclusion body)

Proteins are expressed from plasmid pMH21, 22, 23, pMHS22, pMCH75 in *E.coli* BL21(DE3)(Studier *et al.*,1986). Bacteria was cultured at 37℃ in superbroth(Tryptone 10g, yeast extract 5g, sodium chloride 10g) to which was

added 0.05% $MgSO_4$, 2% glucose, ampicilin 150μg/mL per liter. IPTG induction was at OD_{600} 1.5~2.0 and it was cultured for 3 more hours until OD_{600} 3. Cells were harvested at 3500rpm, 4℃ for 20 minutes and collected in pellet. They were resuspended in 200mL of ice-chilled sucrose solution and they were collected again at 8,000g, 4℃ for 20 minute. The cells were resuspended again in 200mL of ice-chilled water to give an osmotic shock and centrifuged at 15,000g, 4℃ for 20 minutes to gain pellets. This pellet was resuspended in TE buffer(50mM Tris-Cl pH8.0, 20mM EDTA, pH8.0) and treated with lysozyme to remove peptidoglycan layer. The highly concentrated salt 5M NaCl and 25% Triton X-100 was added and mixed evenly with tissuemizer, and incubated for 1 hour and centrifuged at 25,000g for 30 minutes to gain inclusion body pellet. To remove periplasmic protein 25% Triton X-100 was added and mixed evenly with tissuemizer, and centrifuged at 25000g for 30 minutes and Triton X-100 treatment was repeated once again. The pellet was washed with 4M urea buffer(4M urea, 0.1M Tris-Cl). Residual Triton X-100 and urea was removed by resuspending and washing the pellet in TE buffer(50mM Tris-Cl pH7.4, 20mM EDTA pH7.4) and centrifuging at 25,000g, RT for 30 minutes for 3 times. The whole protein quantity of inclusion body gained was analyzed using Coomassie Plus Protein Assay reagent and the antibody-toxin protein quantity was analyzed using Tina 2.0 program. These were stored in −70℃ freezer before the refolding procedure.

(Refolding process and isolation of protein)

Each inclusion body was dissolved in solubilizing buffer solution(6M Guanidine-HCl, 0.1M Tris-Cl, pH8.0, 2mM EDTA pH8.0) and the 5mL solution of the 1:1 molar ratio mixture of B3(Fd)-Ext-PE38 and B3(L) was prepared to be

40mg in 5mL final volume with solubilizing buffer(use the dissolving buffer solution mixing with 40mg antibody-toxin protein and final volume to be 5mL to make.). 0.06mM Dithiotreitol(DTT) was mixed into this for reduction procedure. Refolding procedure was taken in 500mL refolding buffer solution and started with rapidly diluting 5mL of above inclusion body mixture in solubilizing buffer solution, which is a 1:100 dilution ratio. The sample was incubated at 10°C for 48 hours. The quantity of antibody-toxin protein used in this procedure was 80mg and 1L for refolding buffer solution. The refolded protein was taken into dialysis process and isolated through Q-sepharose, source Q, Superdex200 column chromatography(Choe et al., 1994).

(Analysis of cytotoxicity effect against cells)

The cytotoxic effect of isolated protein[B3(Fab)-Ext-PE38]$_2$ was analyzed as described below. Antigen LeY expressing cell lines which are A431, CRL1739, MCF-7 and non-expressing cell line which is KB3-1 were diluted to be $1x10^5$ cells/mL, aliquoted into 96-well plate in 180μℓ/each well and cultured in a CO_2 incubator for 24 hours at 37°C. Purified antibody-toxins are serially diluted to be 10000ng/mL, 1000ng/mL, 100ng/mL, 10ng/mL, 1ng/mL, 0.1ng/mL, 0.01ng/mL in PBS with 0.2% BSA. 20μL of each diluted antibody-toxin was added to 3 wells and the wells were incubated for 24 hours. 1 μCi of [^{3}H]-leucine(NEN) was put into each well, and the cells were cultured for 14 hours. The cells were put in a –70°C freezer and thawed to detach the cells from the plate. The quantity of [^{3}H] incorporated into living cell was analyzed using 1450 Microbeta TriLux Liquid Scintillation Counter(Wallace EG & Co.). The cells are shown in table 2.

(Construction of expression vectors and isolation of inclusion body.)

The nucleotide sequence of pMH21, pMH22, and pMH23, pMHS22 made from PCR was confirmed by nucleotide sequence analysis.

The amounts of proteins in the inclusion body form produced by T7 polymerase system was 100~120mg/L culture for Fd-Ext-toxin, and 80~100mg/ L culture for light chain. To isolate the antibody-toxin protein from whole protein, osmotic pressure was firstly given to the cells to remove outer membrane which releases periplasmic protein and the pellet was cleaned by washing with 25% Triton X-100, 5M NaCl once, washing with 25% Triton X-100 once, washing with 4M Urea(4M urea, 0.1M Tris-Cl) once, and 3 times with TE buffer. Through this process, proteins except inclusion body were removed. The purity of antibody-toxin was analyzed on PAGE gel with densitrometry(TINA 2.0), and the purity of Fd-Ext-toxin and light chain was about 30%.

(Refolding of [B3(Fab)-Ext(4CLFA5X)-PE38]$_2$ molecule)

Each inclusion body, B3(Fd)-Ext-PE38 and B3(L) was used in a 1:1 molar ratio for the refolding process. [Fab-toxin]$_2$ antibody-toxin from prior stuidies, with an uncoupled cysteine for disulfide-bond-bridge at the first amino acid position of Ext(molecule from pCE1), showed a very low production yield after the refolding(Choi *et al.*, 2001). The reason for this low productivity is because of the uncoupled cysteine is too close to the Fab region, causing scrambling with the internal cysteines of the Fab domain. Another reason is that the number of flexible amino acids in Ext is so small(total FA is 7, peptide linker LFA length is 13 in total) that the three-dimensional hindrance between the two big PE38 molecules cannot be overcome. To solve this problem, the uncoupled ~~Cyste~~cysteine was transferred to the 15th position of Ext chain to get far from Fab region and the number of flexible amino acid (FA) of LFA chain was increased to

13(total amino acid number of LFA is 14, the dimer from pCW1) to give room and rotational freedom for effective refolding and dimerization. In this case the disulfide bond is formed between the cysteines on the inserted extension peptide. When the flexible extension peptide is inserted, the productivity of [B3(Fab)-Ext(15CL14FA13)-PE38]$_2$ has increased to 0.06% from 0.014% of the previous [B3(FabH1)-PE38]$_2$ (=[B3(Fab)-Ext(1CL13FA7)-PE38]$_2$. This is because the uncoupled ~~Cystecysteine~~ transferred to the 15th position doesn't cause disulfide bond scrambling with either ~~Cystecysteines~~ inside of Fab or on Ext and this does not cause any interference to the formation of the three dimensional structure. Also the increase of flexible amino acid in LFA makes the three dimensional hindrance between PE38 reduced.

Therefore, in this example the inventors tried to confirm that it is not true that the uncoupled ~~Cystecysteine~~ only at a specific position allows the formation of dimer without interfering interactions with Fab and/or PE38 functional group but that it is true that the uncoupled ~~Cystecysteine~~ on any point in some range of the position enables dimerization too. Also by putting more flexible amino acids in LFA through 5 amino acid increments with the uncoupled Cys fixed at position 4, it was confirmed that the number and the sequence of amino acids in LFA can be varied within a certain range to enable the formation of dimer with various production yields and that a specific number and sequence similar to those of the previous molecules are not required.

The inventors held experiments as below.

1) Comparing the production yield of [B3(Fab)-Ext(4CLFA5X)-PE38]$_2$ according to the LFA chain length and number of flexible amino acids when the Ext chain of Ext(4CL15FA11,4CL20FA16, 4CL25FA16) has 11, 16, 21 flexible amino acids.

2) Examining the effects of [B3(Fab)-Ext(4CLFA5X)-PE38]$_2$ having the

disulfide bond formed by uncoupled ~~Cyste~~cysteine not located at the 1st or 15th but 4th position.

3) Examining the effects of [B3(Fab)-Ext(AQ4CL20FA16)-PE38]$_2$ having AKPCIATQ instead of SKPCISTK in Ext sequence which is derived from the hinge of an antibody.

The results are as followings.

1) When 11,16 or 21 flexible amino acids were inserted, the maximum production yield of [B3(Fab)-Ext(4CLFA5X)-PE38]$_2$ increased to 0.17~0.25%. This is 12.1~17.8 times higher than [B3(FabH1)-PE38]$_2$(=[B3(Fab)-Ext(1CL13FA7)-PE38]$_2$) and 2.8~4.1 times higher than [B3(Fab)-Ext(15CL14FA13)-PE38]$_2$.

Table 4

Plasmid name	Structure of Ext	Yield of dimer(%)	Position of Cys in Ext	Length of L (distance between Cys and F)	Number of GASQEND in L
pCE1	1CL13FA7	0.016	1	13	7
pCW1	15CL14FA13	0.06	15	14	13
pMH21	4CL15FA11	0.18	4	15	11
pMH22	4CL20FA16	0.23	4	20	16
pMH23	4CL25FA21	0.25	4	25	21
pMHS22	AQ4CL20FA16	0.24	4	20	17

According to table 4, one can find the examples of the molecules with LFA peptide, which has 13 to 25 amino acids between the uncoupled ~~Cyste~~cysteine and PE38. Among these, the production yield of [B3(FabH1)-PE38]$_2$(=[B3(Fab)-Ext(1CL13FA7)-PE38]$_2$) which has 13 amino acids is 0.014%, which is nearly no production. However, using 14, 15 amino acids, an insignificant difference from the above 13, allowed the maximum production

yield of the dimer to increase to 0.17~0.25%. This shows that for preventing the hindrance between PE38's, the LFA peptide containing a suitable number of flexible amino acid residues is essential although these LFA peptides between uncoupled ~~Cyste~~cysteine and PE38 are similar. When 11,16,21 amino acids are added to the LFA peptide, there is a small increase in the production yield from 0.17% to 0.25%, indicating the L peptides with flexible amino acids are needed for preventing the hindrance between two PE38. Also, the case of pMH23(B3(Fd)-Ext(4CL25FA21)-PE38) indicates that, even though the 25 amino acid LFA having 21 non-bulky flexible amino acids exists between disulfide bond and PE38, this lengthy LFA did not cause the PE38 functional group to disturb Fab refolding or binding activity and active dimers[B3(Fab)-Ext(4CLFA5X)-PE38]$_2$ were formed.

2) The production yield of [B3(Fab)-Ext(15CL14FA13)-PE38]$_2$ was 0.06% when the disulfide bond is transferred to the 15th location of extension chain but, when the disulfide bond is on the 4th location the maximum production yield increased to 0.17~0.25%. This indicates that the uncoupled ~~Cyste~~cysteine forming disulfide bond doesn't have to take a specific position. The allowable range is sufficiently wide that appropriate disulfide bonds can form without disturbing the formation of tertiary and quaternary structures of neighboring binding domains and functional groups, which are quite massive themselves. This example aimed to determine whether the extension sequence, derived from a natural antibody hinge region, helps the disulfide bonding for dimerization. Our previous results showed that the production yield of [B3(FabH1)-PE38]$_2$(=[B3(Fab)-Ext(1CL13FA7)-PE38]$_2$) having Ext with modified sequence derived from an antibody hinge and uncoupled ~~Cyste~~cysteine was 0.016%. Also the production yield increased to 0.06% for [B3(Fab)-Ext(15CL14FA13)-PE38]$_2$, which has an Ext with a modified sequence derived from an antibody hinge, but

with the uncoupled cysteine in an irregular flexible sequence after the hinge derived sequence. However, if the uncoupled ~~Cyste~~cysteine was put into the middle of the 1st and 15th positions, the production yield was higher than both of the previous 1st and 15th position Therefore whether the hinge sequence derived from an antibody was helpful for dimerization is not clear.

3) For the construction of plasmid pMHS22, the Serine(S) on SKPCISTK sequence which is derived from hinge was exchanged with Alanine(A) that has no –OH group. This is because the –OH group on Serine may make the space sterically too crowded and disturb three-dimensional structure to decrease production yield of dimer. Also, uncoupled cysteine was put into the 4th location and replaced bulky amino acid lysine(K), which occupies a large volume, to a flexible amino acid glutamine(Q) to make AKPCIATQ. The productivities of [B3(Fab)-Ext(4CL20FA16)-PE38]$_2$ by pMH22 having SKPCISTK and [B3(Fab)-Ext(AQ4CL20FA16)-PE38]$_2$ by pMHS22 having AKPCIATQ was similar at nearly 0.2%.

Throughout this experiment, it was clearly demonstrated that the location of the uncoupled cysteine residue in the extension chain for dimerization need not be specific as in naturally coupled ~~Cyste~~cysteines for intra- or inter-chain disulfide bonds and that its position can be varied within a wide range. The relationship between the uncoupled ~~Cyste~~cysteine and dimerization is shown in figure 4.

Also, to decrease three dimensional hindrance between the big functional group PE38, the flexible amino acids are needed following uncoupled ~~Cyste~~cysteine, and according to the experiment the productivity increased with the increased numbers of flexible amino acids. There are 21 flexible amino acids in LFA(flexible chain) for independent refolding of PE38 and Fab, but the LFA did not cause inter-domain hindrance between Fab and PE38 albeit its long length. The formation of [B3(Fab)-Ext(4CL25FA21)-PE38]$_2$ is allowed with an

appropriate disulfide bond between the uncoupled cysteine residues.

(Purification of [B3(Fab)-Ext(4CLFA5X)-PE38]$_2$)

To purify refolded [B3(Fab)-Ext(4CLFA5X)-PE38]$_2$, the fact that PE has net negative charge was exploited and anion exchange resin was used. First, the one liter of dialyzed sample was loaded into Q-sepharose column and the sample was washed and eluted with buffer A and B. The eluted sample was analyzed on 12% reducing and 8% nonreducing SDS-PAGE electrophoresis. The fractions of divalent antibody-toxin was pooled and were gel filtrated using superdex 200 column and eluted with PBS. The final column result is on figure 5.

(Cytotoxicity assay)

Using ^{3}H-incorporation method, the cytotoxicity effect of antibody-toxin was examined on four cancer cell lines. The ADP-ribosylation ability of third domain of PE38 inactivates elongation factorII to inhibit protein synthesis. scFv-PE38 was used as reference molecule for comparison. KB3-1 cancer cell line was used as negative control cell. ID_{50} is the concentration of antibody-toxin when [^{3}H]-leucine-incorporation into cancer cell decreases to 50%. The cytotoxicity assay was held three times each in triplicate samples and the triplicate values were taken average to evaluate the result. The sources of error in triplicate cytotoxicity assays may include sensitive cell conditions, mixing of active and inactive antibody-toxins obtained through *in vitro* refolding and purification, and contaminant proteins. Although the three values are a little different fromone another, they are, however, within the range of experimental errors. The results showed the same or lower ID_{50} values than the reference monovalent molecule, meaning a higher cytotoxicity compared to the reference. This is due to antigen density and structural conditions on the cell surface as observed in the previous case. More knowledge on cell surface antigen structure will make the explanation

possible.

If the cytotoxicity from divalent molecules is superior to monovalent molecules, the treatment effect of a divalent molecule will be higher at the same dosage. Also, the lowest limit of the therapeutic window that is determined by the minimum dosage showing the effect of the drug can be lowered by using the dimer as it has higher binding efficiencies than the monovalent and the same therapeutic effect can be obtained with less amount of dose. This means side effects caused by the functional groups with physiological activities can be overcome by the use of dimers as they can provide the same therapeutic effect with lower doses.

Example 2: The dimerization and effects of antibody-toxin fusion having an extension peptide chain(Ext) with the uncoupled ~~Cyste~~cysteine at the 15th position and a 14 amino acid linker(LFA) with 13 flexible amino acids.

In this example, the B3(Fd)-Ext(15CL14FA13)-PE38 was newly constructed. The flexible peptide $G_4C(G_4S)_2$ was inserted following the fourth Serine on extension chain SKPSISTKASGGPE of B3(Fd)-PE38. The ~~Cyste~~cysteine on sequence derived from an antibody hinge sequence was all changed into Serine that does not form disulfide bond. The composition of B3(Fd)-Ext(15CL14FA13)-PE38 is the same as B3(VH)-B3(CH1)-SKPSISTKASGGGGCGGGGSGGGGSG GPE-PE38. The uncoupled Cys on Ext forms disulfide binds between two B3(Fab)-Ext(15CL14FA13)-PE38 monomers. The uncoupled Cys was located at the 15th position of extension chain to place Cys far from the Fab binding domain whereas in the previous example, the uncoupled Cys was at the 1st position of $[B3(FabH1)-PE38]_2$(=[B3(Fab)-Ext(1CL13FA7)-PE38]$_2$)(protein from pCE1). The goal was to determine

whether the position of Cys has to be specific for disulfide bonding. Also, the inventors were intent on finding out whether the dimerization of B3(Fab)-Ext(15CL14FA13)-PE38 is enhanced by putting 13 flexible amino acids in the peptide linker following the uncoupled Cys. This allows more space between PE38's ~~to~~ decrease the steric hindrance between them during dimerization.

The B3(Fab)-PE38 derived divalent immunotoxin is expected to have more merits than monovalent immunotoxin. First, the binding affinity will be stronger because it's divalent binding valency. Second, cytotoxicity against cancer cells will be better. Third, the stability in circulation of blood will be better. The longer flexible extension peptide chain was applied expecting that [B3(Fab)-Ext(15CL14FA13)-PE38]$_2$ which has longer flexible extension peptide chain has higher productivity and better refolding yield than [B3(Fab)-Ext(1CL13FA7)-PE38]$_2$ having about half number of flexible amino acid in liker L chain.(Choi. *et al.*, 2001)

(Apparatus and Methods)

The construction of B3(Fd)-Ext(15CL14FA13)-PE38 fusion protein was performed by splicing PCR using four primers of the template pMC74(Fd-SKPSISTKASGGPE-PE38 protein expressing vector), $G_4C(G_4S)_2$ which follows after the fourth Serine on SKPSISTKASGGPE sequence between Fd and PE38. The Fd and PE38 fragments from PCR were purified and splicing PCR was performed using the appropriate primers. The products from splicing PCR was purified and they were exchanged with appropriate part of pMC74(Fd-PE38 expressing vector) which was cut with *Nde*I and *Sac*II.

The expression of protein, isolation of inclusion body, refolding, isolation of protein and cytotoxicity assays were performed the same as in example 1.

(Construction of pCW1 Expression vector and isolation of inclusion body)

The structure of pCW1 which expresses B3(Fd)-Ext(15CL14FA13)-PE38 chain composing [B3(Fab)-Ext(15CL14FA13)-PE38]$_2$ is shown on figure 6. B3(Fd)-Ext(15CL14FA13)-PE38 is a modified form of [B3(Fd)-SKPSISTKASGGPE-PE38] using PCR. The peptide chain $G_4C(G_4S)_2$ was inserted between S and G on KASGGPE of [B3(Fd)-SKPSISTKASGGPE-PE38]. The plasmids used are in table 5.

Table 5

Name	Protein Encoded
pMC74	B3(Fd)-SKPSIST-KASGGPE-PE38REDLK
pMC75	B3(L)
pCW1	B3(Fd)-SKPSIST-KAS**$G_4C(G_4S)_2$**GGPE -PE38REDLK

The nucleotide sequence of pCW1 was confirmed through sequencing. The polypeptide was gained from the inclusion body of *E. coli*. The protein purity of inclusion body was measured as 40~60% by densitometry(TINA2.0).

(The refolding of [B3(Fab)-Ext(15CL14FA13)-PE38]$_2$ molecule)

The refolding procedure was performed by mixing the inclusion bodies B3(Fd)-Ext(15CL14FA13)-PE38 and B3(L) in 1:1 molar ratio. The previously reported [B3(Fab)-CKPSISTKASGGPE- PE38]$_2$ (=[B3(Fab)-Ext(1CL13FA7)-PE38]$_2$) immunotoxin which had the uncoupled ~~Cystec~~ysteine at the 1st position of the extension chain to form disulfide bond between two monomers, showed a low production yield after refolding(Choi. et al.,2001). This was because the location of the uncoupled cysteine was too close to the Fab region, and also due to the three dimensional hindrance between the two big PE38s. The solution for this is to transfer the uncoupled ~~Cystec~~ysteine far from Fab and increase the flexible amino acids in LFA to decrease the three-dimensional collision of PE. The designed molecule has an increased number of flexible amino acids in LFA and

more room for the rotational freedom of PE to promote dimerization. The uncoupled ~~Cystecysteine~~ in extension peptide chain forms a disulfide bond to form dimer[Fab-Toxin]$_2$, with the LFA containing 14 amino acids of the GASQEND group. The dimer([B3(Fab)-Ext(1CL13FA7)-PE38]$_2$) containing Fd-CKPSISTKASGGPE-PE38 from pCE1 showing low productivity is composed of 13 amino acids from the point of disulfide bond and the starting point of PE. [B3(Fab)-Ext(1CL13FA7)-PE38]$_2$ (=Fd-SKPSISTKASGGGGCGGGGSGGGGS GGPE-PE38) also has 14 amino acids in the same area but the productivity by refolding procedure was examined as 0.06% which is 4~5 times bigger than the dimer[B3(Fab)-Ext(1CL13FA7)-PE38]$_2$ (=Fd-CKPSISTKASGGPE-PE38) made from pCE1. Therefore, the inserted flexible amino acids increase refolding efficiency by allowing flexible movement of the chain and decreasing three dimensional collisions during dimerization between each B3(Fab)-Ext(15CL14FA13)-PE38.

(Purification of [B3(Fab)-Ext(15CL14FA13)-PE38]$_2$)

The same method was used as example 1. The final column result is shown on figure 7.

(Cytotoxicity assay)

The experiment was performed in triplicate as same as example 1. The ID_{50} of divalent immunotoxin[B3(Fab)-Ext(15CL14FA13)-PE38]$_2$ was measured as 4ng/mL with A431 cell line, 1ng/mL with CRL1739 cell line, 5ng/mL with MCF-7 cell line. The ID_{50}'s measured for the monovalent immunotoxin B3(scFv)-PE40 was high with 5ng/mL for A431, 12ng/mL for CRL1739, 10g/mL for MCF-7. The result for monovalent immunotoxin B3(scFv)-PE40 as a control was exactly the same as reported previously(Brinkmann *et al.*,1991). The ID_{50} of

B3(scFv)-PE40 is appropriate for using it as a reference value because it was measured many times. Also if this value is obtained in the assay it means that the error in the cytotoxicity assay is negligible. The results are shown on figure 8 and table 6.

Table 6

Cell line	Cytotoxicity (ID_{50}; ng/mL) on cell lines of B3 antigen	
	scFv-PE40	[B3(Fab)-Ext(15CL14FA13)-PE38]2
A431	5	4
CRL1739	12	1
MCF7	10	5
KB3-1	>1000	>1000

These results show that the divalent immunotoxin[B3(Fab)-Ext(15CL14FA13)-PE38]$_2$ has 12 times the higher cytotoxicity than the monovalent B3(scFv)-PE40 with CRL1739 cell line.

The CRL1739 cell line that showed a 12-fold difference in the cytotoxicity assay is a stomach cancer cell line. This means that the divalent immunotoxin has more cytotoxicity depending on the surface structure against cancer cells that express the same LeY antigen. If LeY is on a very long and flexible structure and binding of divalent immunotoxin to two LeY antigens at same time is easy, divalent immunotoxin will bind two of LeY simultaneously showing more binding affinity than monovalent immunotoxin. If LeY is on a non-flexible structure or LeY's are too far apart from each other, then the binding will be the same for both divalent and monovalent immunotoxin as the binding of the immunotoxin to antigen is through only one binding domain to one antigen even though the molecule has divalent binding domain. In the case of cell line CRL1739, it seems that it has LeY on a very long and flexible polysaccharide structure and allows [B3(Fab)-Ext(15CL14FA13)-PE38]$_2$ to bind two antigens simultaneously. The binding of an antibody with antigens on cell surface can

stimulate the growth of the cell depending on their interaction. Some cell growth was observed at very high concentration of divalent immunotoxin (data not shown) but not for monovalent immunotoxin. The previously reported monovalent B3 immunotoxin has yet to be known to stimulate cell growth by the antigen-antibody interaction. The different effects of monovalent and divalent immunotoxins on cell growth remain to be investigated.

Example 3: The dimerization of an antibody-toxin fusion that has extension peptide chain(Ext) with uncoupled ~~Cystec~~ysteine at the 1st position and a 13 amino acid linker(LFA) containing 7 flexible amino acids

The B3(Fab) and PE38 are used in constructing divalent immunotoxin [B3(FabH1)-PE38]$_2$ (=[B3(Fab)-CKPSISTKASGGPE-PE38]$_2$)(=[B3(Fab)-Ext(1CL13FA7)-PE38]$_2$). The divalent immunotoxin B3 has about 174.4kDa of molecular weight and comprises two light chain of B3 and two chains composed of B3(Fd) fused with PE38. There are three cysteine residues in the hinge sequence of a B3 antibody. The two C-terminal cysteine residues are changed to serines and only the N-terminal cysteine is used to make the Ext chain. It forms disulfide bond between monovalent immunotoxin to produce dimer.

(Materials and methods)

The methods used are same as example 1. MTT(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) from Sigma Co. was used at 1.5mg/mL final concentration. Cy5TM AutoReadTM sequencing kit for sequence analysis was a product from Pharmacia Biotech. The plasmids used are shown on table 7.

Table 7

Plasmid Name	Protein Encoded

pMC75	B3(L)
pCE1	B3(Fd)-CKPSISTKASGGPE-PE38
pMC74	B3(Fd)-SKPSISTKASGGPE-PE38
pMC76	B3(L)-KASGGPE-PE38

The method using chromatography column was the same as example 1.

(Construction of plasmid pCE1 expressing B3(FdH1)-PE38=B3(Fd)-Ext(1CL13FA7)-PE38)

Plasmid pCE1(expressing B3(FdH1)-PE38) is a modified form of pMC74(expressing B3(Fd)-PE38=B3(Fd)-SKPSISTKASGGPE-PE38). PCR was performed to change the sequence to pCE1. (pCE1 expresses B3(FdH1)-PE38=B3(Fd)-CKPSISTKASGGPE-PE38).

The constructed expression system was confirmed by DNA sequence analysis. Primers used were designed appropriately to be used with each template DNA and to code designed amino acid sequence. B3(L) is expressed from pMC75.

(Preparation of protein)

The methods for expression of B3(FdH1)-PE38 and B3L, purification of spheroplast and inclusion body, quantity analysis of inclusion body, refolding procedure, purification of B3(FabH1)-PE38 and $[B3(FabH1)-PE38]_2$ were same as example 1.

(Measurement of $[B3(FabH1)-PE38]_2$ formation according to temperature change)

$[B3(FabH1)-PE38]_2$ formation from B3(FabH1)-PE38 monomer isolated by Mono-Q was measured at 37℃, 40℃, 45℃, 53℃ incubating for 24hours. $[B3(FabH1)-PE38]_2$ was purified with Mono-Q and proteins of each fraction were analyzed with electrophoresis.

(Measurement of [B3(FabH1)-PE38]$_2$ formation using cross-linkers)

B3(FabH1)-PE38 obtained from TSK-GEL G3000SW and cross-linkers, which are bis-maleimidohexane(BMH) and 1,11-bis-maleimidotetraethyleneglycol (BM[PEO]$_4$), were reacted together in molar ratio of 6:1, 5:1, 4:1, 3:1, 2:1, 1:1, 1:3 in reaction buffer to connect the Thiol(-SH) groups of ~~Cystecysteine~~ of two B3(FabH1)-PE38. The proteins were analyzed by electrophoresis.

(Cytotoxicity assay of purified proteins against cancer cells)

The same method as example 1 was used except the following: The viability of cancer cells was measured according to decomposition of MTT to analyze the cytotoxic effect of purified B3(FabH1)-PE38 and [B3(FabH1)-PE38]$_2$. Cancer cells were cultured with immunotoxins and 20μL of 5mg/mL MTT was added. The samples were wrapped with aluminum foil and left alone in a 5% CO_2 incubator for 10 hours at 37°C. The reduced MTT-formazan was centrifuged at 3000rpm for 4~15 minutes to make MTT-formazan crystal to pellet and 200μL of the supernatant was removed. 100μL of 0.016N acidic isopropanol was added. A microfilter plate shaker was used at 300rpm to melt MTT-formazan and the optical density was measured using ELISA READER at 570nm. Results were average values of three samples.

(Purification of B3(FabH1)-PE38 and [B3(FabH1)-PE38]$_2$)

The same method as example 1 was used.

The quantity of B3(FabH1)-PE38 and [B3(FabH1)-PE38]$_2$ was 4.3mg and 16.5mg, respectively, which is 3.8% and 0.016% of the total quantity of B3(FdH1)-PE38 and B3L used in refolding. The reason for the low productivity

of the dimer [B3(FabH1)-PE38]$_2$ is that a large amount of B3(FdH1)-PE38 and B3L coagulate and precipitate to disappear during refolding and dialysis. Also the reason for the low productivity of [B3(FabH1)-PE38]$_2$ in spite of 3.8% B3(FabH1)-PE38 produced, may be that the cysteine is located in a place difficult for forming disulfide bonds due to unfavorable interactions of Fab and/or PE38 or it is in a wrong orientation or is buried inside the three-dimensional structure. But the small quantity of [B3(FabH1)-PE38]$_2$ obtained in this example was assumed to have dimerized via the rare disulfide bond formation before the complete refolding between small amounts of B3(FdH1)-PE38; this disulfide bond may be able to form before the disturbance on its formation by Fab and PE38, followed by a second disulfide bond with B3L to produce dimers.

(Measurement of [B3(FabH1)-PE38]$_2$ formation according to temperature change)

Because the productivity of [B3(FabH1)-PE38]$_2$ is very low, the formation by disulfide bond between B3(FabH1)-PE38 was checked with heating the monomer B3(FabH1)-PE38 to relax the structure of the monomer and to free the ~~Cyste~~cysteines from the unfavorable interactions of Fab and/or PE38 and to allow the oxidation for disulfide bond formation. Productivity of [B3(FabH1)-PE38]$_2$ was highest at 45℃ heating observed with B3(FabH1)-PE38 monomer isolated from Mono-Q. But many other kinds of side products besides [B3(FabH1)-PE38]$_2$ was formed too.

(Measurement of [B3(FabH1)-PE38]$_2$ formation using cross-linkers)

To increase productivity of [B3(FabH1)-PE38]$_2$, the cross-linkers(BMH and BM[PEO]$_4$) was used to connect the –SH group between two B3(FabH1)-PE38. The formation of [B3(FabH1)-PE38]$_2$ was hardly observed when the molar ratio of B3(FabH1)-PE38 and cross-linker was used at 6:1, 5:1, 4:1, 3:1, 2:1, 1:1,

1:3 in the reaction. This result shows when the refolding with cross-liker was performed, the ~~Cyste~~cysteines for disulfide bond still cannot get close to each other to be linked by the cross-liker because of unfavorable interactions with Fab and/or PE38, or the cysteine being in a wrong orientation, or the cysteine being buried in the three-dimensional structure, or because the lysine which follows after cysteine is so bulky that it interrupts the bonding between cross-linkers and ~~Cyste~~cysteines. This leads to the failure in forming a dimer between two monomers.

(The cytotoxicity assay of purified proteins on cancer cells)

The cytotoxicity effect on cancer cells of B3(FabH1)-PE38 and $[B3(FabH1)\text{-}PE38]_2$ can be observed by measuring viability of cancer cells which is indicated by the amount of MTT-tetrazolium being reduced to MTT-formazan by the mitochondrial dehydrogenase. The cancer cells used were A431, MCF7, CRL1739, which have B3 antigens, and KB3-1 as a negative control, which doesn't have B3 antigen. B3(scFv)-PE40 is a single chain immunotoxin, which is a monovalent molecule, and it was used as reference molecule. The ID_{50} which shows cytotoxic effect of B3(scFv)-PE40, B3(FabH1)-PE38 and $[B3(FabH1)\text{-}PE38]_2$ was 61.3ng/mL, 30ng/mL, 10.3ng/mL respectively. However, MTT-tetrazolium has to be delivered to the mitochondria in the MTT assay and it may introduce errors depending on the conditions of each cancer cells. But B3(FabH1)-PE38 and $[B3(FabH1)\text{-}PE38]_2$ had 2 times and 6 times higher effects than B3(scFv)-PE40 respectively. However, the reference molecule showed very low cytotoxic activity compared to the previous reports reported as 2~5ng/mL ID_{50}. This is because the cytotoxicity assay is very sensitive to conditions of the cells and the immunotoxins were not purified sufficiently, containing impurities. As the errors of MTT assay is relatively large, the reconfirmation of the results is

preferred.

In conclusion,

1) B3(FdH1)-PE38 and B3L occupied 17~25% of the total expressed protein in the cell when they were over-expressed.

2) Production yield of B3(FabH1)-PE38 was 4.3mg which is 3.8% and $[B3(FabH1)\text{-}PE38]_2$ was 16.5μg which is 0.016% when prepared with B3(FdH1)-PE38 and B3L in 82mg and 32.2mg amounts respectively in the refolding procedure.

3) The productivity of proteins with the correct conformation formed through the 100-fold rapid dilution refolding procedure is very low, and 96% of the protein were incorrectly refolded and aggregated.

4) The highest productivity was obtained at 45℃ when $[B3(FabH1)\text{-}PE38]_2$ was formed by heating B3(FabH1)-PE38 monomer.

5) B3(FabH1)-PE38 does not form dimers by connecting the monomer with long cross-linkers like BMH and $BM[PEO]_4$.

6) The ~~Cyste~~cystein residues used for disulfide bonding between B3(FabH1)-PE38 are placed in an environment difficult for forming dimers.

7) The purified B3(FabH1)-PE38 and $[B3(FabH1)\text{-}PE38]_2$ prepared from large amount of materials showed 2 times and 6 times higher cytotoxicity respectively than the B3(scFv)-PE40 reference molecule.

8) For high purity preparation of $[B3(FabH1)\text{-}PE38]_2$, large amount of materials are needed and the contaminating proteins have to be removed.

Example 4: The dimerization of an antibody-toxin having an extension peptide chain(Ext) with uncoupled the cysteine at the 1st, 4th or 6th position and an affinity domain containing a flexible liker peptide (LADFA)

comprising the CH2, CH3 self-affinity domains of an antibody with the $(G_4S)_2$ sequence.

In this experiment, three uncoupled cysteine residues were positioned at the frontal region of extension chain, and they form a very thermodynamically stable dimer with three disulfide bonds. In other words, this is to test whether the large sequence with an Fab and a bulky PE38 group with additional native internal cysteine residues, can refold into the correct conformation without being scrambled with three uncoupled cysteines of the extension chain. In another words, this is to confirm whether a binding domain-functional group fusion dimer can form a highly thermodynamically stable structure with triple disulfide bonds when the extension chain connecting binding domain and functional group contains multiple uncoupled cysteines. Predicting whether the multiple uncoupled cysteines will find their disulfide bond couple correctly without interference from or intermixing with neighboring bulky sequences as to form a dimer is not easy forthe skilled artisan.

[B3(Fab)-h(H123-CH2/CH3/Fc)-PE38R]$_2$ having Ext(LADFA) was produced which has Fc, CH2, CH3 domain in LFA sequence having self-affinity for easy meeting of the ~~Cystec~~ysteines for dimerization and flexible amino acid sequence following the 'self-affinity domain'. The CH3 domain was reported not to disturb antigen binding and induces homodimerization (Acpua *et al.*, 1998, Ridgway *et al.*, 1996). Also, the molecule with the CH3 domain has a similar antigen binding affinity and homodimerization ability as those with the Fc domain which has both the CH2 and CH3 domains (Alt *et al.*, 1999). Therefore, the merit of stability of Fab-toxin in blood circulation is saved and also the production yield of dimer[Fab-toxin]$_2$ has increased through the insertion of the Fc, CH2, CH3 domains inducing homodimerization(Wu *et al.*, 2001). Also, the binding affinities

of the divalent and monovalent molecules were compared (Gall *et al.*, 1999).

(Materials and Methods)

E.coli BL21(DE3) was used for protein expression system.

For the construction of plasmid having Fab-h(H123-CH2/CH3/Fc)-PE38 chain gene, Fab-PE38 was obtained from pMC74 as a template and Fc region was obtained from human hinge(including three uncoupled ~~Cyste~~cysteines) and Fc containing pcDNA3Cγ1 as a template. For the light chain, 5'-end 6xHis tagged chain from pMCH75 was used. Each name and construction procedure of the plasmid is on figure 12,13,14 and table 8.The same media, reagents, enzymes, columns as example 1 are used.

Table 8

Name	Proteins Encoded
pLSC52	B3(Fd)-human(H123-Fc)-$(G_4S)_2$-KASGGPE-PE38REDLK
pLSC32	B3(Fd)-human(H123-CH3)-$(G_4S)_2$-KASGGPE-PE38REDLK
pLSC22	B3(Fd)-human(H123-CH2)-$(G_4S)_2$-KASGGPE-PE38REDLK
pMC74	B3(Fd)-SKPSIST-KASGGPE-PE38REDLK
pMCH75	H6-B3(L)

(Construction of plasmid and Preparation of protein)

To construct pLSC52 which contains all of the Fc, PCR of the region from the hinge to Fc using pcDNA3Cγ1 as a template was performed. A PCR of the Fd region from pMC74 was also performed and a splicing PCR was performed with these two fragments. The produced fragments and pMC74 were digested with *Nde*Iand *Hind*III for insertion ligation. Plasmids with CH2, CH3 were constructed the same way and called pLSC22 and pLSC32, respectively.

The methods for expression and isolation of protein and protein refolding

procedure were the same as example 1. The molar ratio of LSC52 or LSC32 or LSC22 to MCH75 was 1:1 and the quantity of IT protein added was 40mg/5mL for the condition of 500mL refolding and the method was same as example 1. The method for purification of refolded antibody-toxin was the same as example 1.

The method for cytotoxicity assay on 4 cell lines with [B3(Fab)-h(H123-Fc)-PE38]$_2$, [B3(Fab)-h(H123-CH3)-PE38]$_2$, [B3(Fab)-h(H123-CH2)-PE38]$_2$, [B3(Fab)-h(H123-CH2)-PE38] isolated from Superdex 200 was the same as example 1.

(Production and purification of IB protein)

To produce [B3(Fab)-h(H123-CH2/CH3/Fc)-PE38]$_2$, plasmids pLSC22, pLSC32, pLSC52 encoding [B3(Fd)-h(H123-CH2/CH3/Fc)-PE38]$_2$ were constructed and pMCH75 was used for light chain.

The inclusion body protein preparation from T7 polymerase mass production system was repeated 12 times to get a 38.2mg/liter culture in average. Measured by densitometry analysis(Tina2.0), the purity of protein on the PAGE gel was 34.2% for the heavy chain which was the average of 9 measurements and 40.9% for light chain which was the average of 3 measurements. The inclusion body was analyzed through SDS-PAGE. To enhance the purity of the inclusion body, additional washing procedure treating with 4M Urea buffered by Tris-Cl pH7.4 for 3 hours was performed in addition. The purity of inclusion body was over 30% and the protein folding products were observed.

(Refolding of proteins)

Disulfide bonds during refolding can be formed in two different ways: intra-chain disulfide bonds and inter-chain disulfide bonds. B3(Fd)-h(H123-CH2/CH3/Fc)-PE38 from pLSC22, pLSC32, pLSC52 and light chain from

pMVH75 form a disulfide bond between Fd and L in the refolding solution to assemble an Fab domain. Disulfide bonds are formed between the H123 ~~Cyste~~cysteine on two of B3(Fd)-h(H123-CH2/CH3/Fc)-PE38 monomer to make a dimer molecule.

(Purification of refolded antibody-toxin)

After its purification, the yield of [B3(Fab)-h(H123-Fc)-PE38R]$_2$, having Fc domains, was the lowest value because the molecule size is relatively large(divalent: 228kd) and the refolding is easily disturbed. [B3(Fab)-h(H123-Fc)-PE38R]$_2$ which has both the CH2 and CH3 domains had a dragging bands besides the right size refolded band compared to those molecules with only one domain. Since the size of [B3(Fab)-h(H123-Fc)-PE38R]$_2$ is so large, incorrectly folded molecules accumulate during refolding.

[B3(Fab)-h(H123-CH3)-PE38R]$_2$ which has a CH3 domain did not show the peaks of divalent and monovalent molecule separated on Superdex 200 column. This is because they exist as a divalent form while being isolated through superdex200 column due to the strong self-affinity of CH3 domain. But during SDS-PAGE, those dimer molecules formed only by CH3 affinity but not by covalent disulfide bonds fall apart and are observed as monomers on the SDS-PAGE. According to the band intensity, the dimers formed by disulfide bonds on the Ext or CH3 affinity are in similar quantities. Therefore the CH3 region is effective for dimerization of the Fab molecules, keeping them in close proximity, and their refolding with dimer formation will take place even though the number of uncoupled cysteine residues is 3. The immature disulfide bonds within the affinity domain could be matured by a long exposure to atmospheric oxygen. The immature disulfide bonds are well known to the skilled artisan.

Finally, the quantity of divalent [B3(Fab)-h(H123-CH2)-PE38R]$_2$ having

a CH2 is small but that of monovalent ones is large. This shows that CH3 self affinity is much stronger than CH2 self affinity to form more dimers. Through known facts that the self affinity of CH2 is very weak and through experimentally observed facts that monomers with a single CH2 domain formdimers in very small quantities, it can be deduced that dimers can form through disulfide bonds even with three uncoupled cysteines in the extension chain without the assistance from the affinity domain.

(Cytotoxicity assay)

The cytotoxic effect of the purified immunotoxin[B3(Fab)-h(H123-CH2/CH3/Fc)-PE38]$_2$ was tested on 4 cancer cell lines. According to cell types and culture conditions, the effect of divalent and monovalent molecules differed.

(Productivity of dimerization)

Table 9

Refolding	[B3(Fab)-h(H123-Fc)-PE38]$_2$	[B3(Fab)-h(H123-CH3)-PE38]$_2$	[B3(Fab)-h(H123-CH2)-PE38]$_2$
1st (240mg/3L)	148g 0.06%	455g 0.19%	198g 0.08%
2nd (80mg/1L)	44g 0.05%	146g 0.18%	78g 0.10%
3rd 80mg/1L)	47g 0.06%	162g 0.20%	59g 0.07%

In the case of [B3(Fab)-Ext(15CL14FA13)-PE38]$_2$, which has an extension peptide chain SKPSISTKASG$_4$C(G$_4$S)$_2$GGPE between Fab and PE38, the Ext(15CL14FA13) has decreased the steric hindrance between PE molecules, and the productivity of this molecule was 0.06%.

In this experiment, dimerization was induced using the CH3 affinity domain and the productivity was 0.19%. This is three times higher than [B3(Fab)-Ext(15CL14FA13)-PE38]$_2$. The productivity using the CH2 domain was 0.08%, and the Fc domain 0.06%. This is due to the difficulties in refolding of the self

affinity region and weak affinity of CH2. These are similar results to that of [B3(Fab)-Ext(15CL14FA13)-PE38]$_2$, and it means that in these two cases the dimer production was not improved. However a skilled artisan can expect a good productivity if he employs smallersized, stronger affinity domains. Especially, in the case of [B3(Fab)-h(H123-Fc)-PE38R]$_2$ which has a CH3 domain, the productivity was 0.06% because the size was too big for the proper folding.

According to the results above, putting self-affinity domains in LFA induces assembly between uncoupled cysteines, and the disulfide bond formation even with three ~~Cystecysteines~~ can take place normally even with 3 cysteine residues without disturbing the 3-dimensional conformation of the binding domain and functional group domain. And also, this triply disulfide bonded dimer is more stable at room temperature when it is left alone and more resistant to damage by proteinases than singly disulfide bonded dimers.

Example 5: The dimerization of [B3(Fab)-cytosine deaminase]$_2$ having the Fab of B3 antibody as a binding domain and cytosine deaminase as a functional group.

Up to now, monoclonal antibodies, monoclonal antibody treatments, toxins and radionuclide complexes and others have been studied for many years. In recent years, many treatments based on monoclonal antibodies were clinically approved (Rituxan, Herceptin, Panorax). And some other treatments are in clinical demonstration.

In order to use big molecules as successful therapeutic agents for solid tumor, problems such as transmission hindrance into tumor mass, foreign antigen, efficacy of complex treatment and efficient transfer from monoclonal antibody into cancer cell must be overcome.

An alternative plan for therapeutic agent delivery is to undergo two steps for cancer treatment. Once the complex binds to a cancer cell and gets removed from blood circulation, an enzyme activates an anti-cancer prodrug to activate it. The isolated drug can be transmitted into tumor mass and eliminates both monoclonal antibody binding cells and non-binding neighboring cells.

This treatment is called Antibody-Directed Enzyme Prodrug Therapy(ADEPT) which is a new method for selective cancer treatment. It introduces enzyme-antibody complex to change harmless prodrug at a cancer cell surface selectively into a cytotoxic compound. Bagshawe first reported this idea at 1987.

In the first step of ADEPT, antibody-enzyme complexes accumulate on cancer cells. As time passes, the complex gets degraded in blood and normal tissues. The second step is to introduce harmless a prodrug and transforming it into a cytotoxic drug by an enzyme from the complex.

Through this method, one can improve cancer specificity and deliver higher doses of drugs than direct administration. Also, one molecule of enzyme can amplify cytotoxicity by catalyzing many prodrugs into activated drugs, and since these activated drugs have small molecular weights, they easily diffuse to cancer cells nearby. As a bystander effect, a unique character of ADEPT, the cells near the cancer cells also get killed.

There are various monoclonal antibodies, enzymes and prodrugs that can be applied to ADEPT. Among enzymes there is yeast cytosine deaminase which catalyze exchange of cytosine into uracil and it can change anti-mold reagent 5-Fluorocytosine(5-FC) into 5-Fluorouracil(5-FU) as an anti cancer agent. Especially the yeast cytosine deaminase is reported to have more remedial value than bacterial enzymes used in enzyme-prodrug treatment. In addition, yeast cytosine deaminase prevents prodrug activity by restriction enzymes in blood or

cells because it's from non-mammal, which has no homology to mammals. And it can be used easily with large amount because it has no translational modifications on it.

Pharmaceutical efficacy and toxicity must be clinically demonstrated before use and this is the dormant problem of applying ADEPT. However, 5-FU, which is used present clinically, can be used at colon carcinoma, a difficult cancer to treat using other chemical agents. And the cytotoxicity of the drug and prodrug was confirmed in H2981 human lung adenocarcinoma cells. It was proved that there was no cytotoxicity in 5-FU, which has an ID_{50} of 20 μM compared to 5-FC, which has no effect even at the concentration of 200μM.

In this experiment, the recombinant protein [B3(Fab)-cytosine deaminase]$_2$ for ADEPT application was constructed. This molecule was made by modifying Fab to create a divalent molecule and keeps the structural stability and the turnover rate of Fab. The second disulfide bond in the hinge was used for dimerizing monovalent Fab-enzyme to the divalent molecule. Since this molecule is divalent, the binding affinity will be more than 2 times stronger and since the enzyme quantity has increased two times, the cytotoxicity will increase more. Also, although the IgG didn't have complete free rotation ability due to the three disulfide bonds of Fab region on the hinge, [B3(Fab)-enzyme]$_2$ has free rotation ability on its binding domain, and it will lead to stronger binding to antigens spread over the cells.

Though [B3(Fab)-cytosine deaminase]$_2$ is a big molecule, it has a strong equilibrium binding affinity, fast binding reaction rate, increase in enzyme administration and a long active turnover rate in the blood circulation system, and it will show a higher efficacy than Fv derivatives.

(Apparatus and methods)

The same method was used as example 1. For the construction of plasmid containing B3(Fd)-yCD gene, chromosomal DNA from *Saccharomyces cerevisiae* was used for template and pMC74 was used as template for Fab region. For the light chain, pMCH75, which is 6x His tagged on 3'-end was used as a template. Mediums, reagents, enzymes and columns were used the same as example 1. Constructed plasmids are shown on table 10.

Table 10

Plasmid	Protein Sequence
pKL1	H6-CDase
pKL2	B3(Fd-SKPCIST-KAS-$(G_4S)_2$-GGPE-CDase-H6
pKL3	B3(Fd-SKPCIST-KAS-$(G_4S)_2$-GGPE-CDase
pKL4	H6-B3(Fd-AKPCIAT-QAS-$(G_4S)_2$-GGPE-CDase

(Construction of plasmids)

A plasmid which expresses B3(Fd)-yCD fusion protein was constructed from PCR using the CDase containing region of *Saccharomyces cerevisiae* chromosomal DNA as a template and the two primers below. The CDase was purified and recombination with pMC74 led to recombinant plasmid pKL1.

Primer 1: 5'-GGC-CCA-TAT-GCA-TCA-CCA-TCA-CCA-TCA-CGT-GAC-AGG-GGG-AAT-G-3'

Primer 2: 5'-TTG-GTT-TGA-AGA-TAT-TGG-TGA-GTA-GGA-ATT-CGG-CC-3'

Primer 1 binds to 5'-end of CDase. Primer 2 binds to 3'-end of CDase to be used in CDase purification by PCR.

From PCR using pKL1 as a template with two primers below, CDase fragment was gained and it was recombined with PE of pMC22 to produce pKL2.

Primer 3: 5'-GGC-CCC-CGA-GGT-GAC-AGG-GGG-AAT-G-3'

Primer 4: 5'-GAA-GAT-ATT-GGT-GAG-CAT-CAC-CAT-CAC-CAT-CAC-TAG-GAA-TTC-GGC-C-3'

Primer 3 binds to 5' end of CDase. And primer 4 having six Histidines binds to 3'end of CDase to be used in CDase purification by PCR.

pKL2 has B3(Fd)-SKPCISTKAS-GGGGSGGGGS-GGPE-CDase-6His.

CDase region from pKL1 and CDase region from pKL2 was recombined to produce pKL3. pKL3 is B3(Fd)-SKPCISTKAS-GGGSGGGS-GGPE-CDase and doesn't have six His at the 3' end.

pKL4 was constructed as following. PCR was performed by using template pMC74 and two primers below to gain Fd fragment which has six His on 5'-end and recombined with pKL3 to produce pKL4.

Primer 5: 5'-GGC-CCA-TAT-GCA-TCA-CCA-TCA-CCA-TCA-CGA-TGT-GAA-GCT-GGT-GGA-GTC-T-3'

Primer 6: 5'-GGG-AAT-TCA-TTA-AGC-TTG-TGT-AGC-TAT-GCA-AGG-CTT-AGC-ACC-ACA-3'

Primer 5 has six His and binds to Fd region. Primer 6 has AKPCIATQAS and binds to 3' end of Fd.

The nucleotide sequence of plasmid pKL4 was confirmed by ALFexpress Dedeoxy Sequencing Kit(Amersham Pharmacia).

The method for protein expression and isolation, protein refolding, purification of refolded antibody-toxin was the same as example 1.

~~The cytotoxicity assay was performed on 4 kinds of cell lines with $[B3(Fab)\text{-}CDase]_2$ isolated by Superdex200 and the same method was used as example 1.~~

~~As in the preceding methods, we were able to purify molecules having a normal binding activity and active functional groups. And the binding activity of the binding domain and cytosine deaminase activity of the functional domain of the purified molecule was confirmed to be normal.~~

Example 6: The dimerization and the increase in the length of flexible chain(LFAhaving the uncoupled cysteine fixed at the 4th position of the extension peptide chain(Ext).

The inventors increased the length of peptide linker(L) which runs from the uncoupled Cys on Ext to functional group to find the relation between the linker length and dimerization. The peptide linker(L) must be long enough to give room for the refolding to occur without disturbance from the steric collision between two functional groups when a dimer forms between two monovalent binding domain-functional domain fusions. Also, the peptide linker, composed of flexible amino acids, must have a good flexibility to allow the functional groups to become separated wide enough in an appropriate angle. But if the peptide linker is too long, the uncoupled ~~Cystecysteine~~ on Ext will have too much freedom to move and encounter the other naturally coupled ~~Cystecysteines~~ of in intra-chain and inter-chain disulfide bonds, leading to disulfide bond scrambling and inactivation of the molecule. And also since the peptide linker does not have a definite structure, it might penetrate into structures of the binding domain or functional group during refolding to disrupt their correct three dimensional structure.

To find the relation between the length of the peptide linker and dimerization, plasmids were constructed as below.

pMH28: B3(Fd)-SKPCG----------PE38; Ext(4CL1FA1)

pMH29: B3(Fd)-SKPCKASPE----------PE38; Ext(4CL5FA3)

pMH30: B3(Fd)-SKPCISTKASGGPE----------PE38; Ext(4CL10FA6)

pMH34: B3(Fd)-SKPCISTKAS$(GGGGS)_4$GGPE--PE38; Ext(4CL30FA26)

pMH35: B3(Fd)-SKPCISTKAS$(GGGGS)_5$GGPE--PE38; Ext(4CL35FA31)

pMH36: B3(Fd)-SKPCISTKAS$(GGGGS)_6$GGPE--PE38; Ext(4CL40FA36)

pMH37: B3(Fd)-SKPCISTKAS$(GGGGS)_7$GGPE--PE38; Ext(4CL45FA41)

pMH38: B3(Fd)-SKPCISTKAS$(GGGGS)_8$GGPE--PE38; Ext(4CL50FA46)

The protein chains above have a Cys residue in LFA at the 4th position of Ext. Each peptide linker(L) has 1,5,10,30,35,40,45 or 50 amino acids with 1,3,6,26,31,36,41 or 46 non-bulky flexible amino acids, respectively.

The eight molecules of $[B3(Fab)\text{-}Ext(4CLxxFAxx)\text{-}PE38]_2$ type in this example were made to have the same potential for steric hindrance and intermixing that can be caused by the uncoupled Cys by fixing the Cys at 4th position on the Ext chain. And the amino acids following the uncoupled Cys were kept the same except the numbers of flexible amino acid was increased in five amino acids step to increase the peptide linker length and the sterical space was expanded with the same step. Among these molecules, the molecule with the shortest L having 1 amino acids which is Ext(4CL1FA1) and the longest L having 50 amino acids which is Ext(4CL50FA46) showed very low productivity.

Therefore, this example shows that when the peptide linker(L) has over 50 amino acids, it is impossible to obtain dimers formed from the disulfide bond bridge between the two fusion monomers comprising a 50kD Fab domain of B3 antibody and a 38kD functional group PE38.

(Materials and methods)

The same materials and methods were used as example 1.

(Construction of plasmids)

The expression plasmids were constructed by PCR using primers which are appropriate for each DNA sequence. Used templates were the same as example 1 and the same plasmids were used as example 1. The procedure for

inserting GGGGS sequence repeatedly was done by restriction and ligation.

(Protein expression and isolation of inclusion body)

The same methods were used as example 1.

(Refolding procedure and isolation of proteins)

The same methods were used as example 1.

(Construction of expression vector and isolation of inclusion body)

The coding nucleotide sequences obtained through PCR and cloned in expression plasmids was confirmed by sequence analysis.

The proteins in the form of inclusion body was analyzed by densitometry analysis(TINA2.0) on PAGE sample and the purity of protein chain was in 25~30%.

(Refolding of [B3(Fab)-Ext(4CLFA5X)-PE38]$_2$ molecule)

Table 11

Plasmid name	Structure of Ext	Dimer Yield(%)	Position of Cys in Ext	Length of L (distance bet. Cys and F)	Number of GASQEND in L	Note
pMH28	Ext(4CL1FA1)	<<0.01	4	1	1	
pMH29	Ext(4CL5FA3)	0.01	4	5	3	
pMH30	Ext(4CL10FA6)	0.04	4	10	6	
pCE1	Ext(1CL13FA7)	0.016	1	13	7	Example3
pCW1	Ext(15CL14FA13)	0.06	15	14	13	Example2
pMH21	Ext(4CL15FA11)	0.18	4	15	11	Example1
pMH22	Ext(4CL20FA16)	0.23	4	20	16	Example1
pMH23	Ext(4CL25FA21)	0.25	4	25	21	Example1

pMHS22	Ext(AQ4CL20FA16)	0.24	4	20	17	Example1
pMH34	Ext(4CL30FA26)	0.32	4	30	26	
pMH35	Ext(4CL35FA31)	0.17	4	35	31	
pMH36	Ext(4CL40FA36)	0.21	4	40	36	
pMH37	Ext(4CL45FA41)	0.08	4	45	41	
pMH38	Ext(4CL50FA46)	<<0.01	4	50	46	

According to table 11 above, molecules with very long peptide linkers and very short ones have very small production yields. It is also possible to detect them but their quantities are not enough for testing. It also shows that the length of the peptide linker do not have a fixed relationship with the production yield of dimers, and that it is possible to obtain dimers within some range of the peptide linker_length. It can be assumed that the large-sized Fab and PE38 do not get disturbed during refolding even if the length of the peptide linker is long. This example, however, shows that if the number of amino acids in the peptide linker is increased to more than 50, the dimers can be detected but it is not practical to prepare them.

This means that using more than 50 amino acids in the peptide linker is not realistic. If the protein production technology develops, the purification of dimers with a 50 amino acid peptide linker may become feasible

(Purification of B3(Fab)-Ext(4CLFA5X)-PE38$_2$)

It was analyzed the same as example 1 and the purity was confirmed on PAGE.

~~(Cytotoxicity assay)~~

~~The cytotoxicity assay was performed in triplicate. The result was that some molecules had same ID_{50} value as monovalent molecule and some had higher cytotoxicity effect than monovalent molecules in the previous example.~~

~~This is due to the density and the structural conditions of antigens on the cell surface according to the cell type.~~

Example 7: Dimerization when the uncoupled ~~Cyste~~cysteine is on the 25th, 35th, 45th position on the extension chain(Ext)

The relationship between dimerization and the location of uncoupled Cys residues at the 25th, 35th and 45th positions of Ext was observed. The uncoupled ~~Cyste~~cysteine on Ext between binding domain and functional domain can react and become oxidized to form disulfide bonds with the naturally coupled Cys residues originally forming natural intra-chain and inter-chain disulfide bonds of the binding domain and functional domain. This can cause the scrambling of the disulfide bonds and ruin the structures of domains to inactivate them. The position of uncoupled Cys is a very important factor that plays a role in the scrambling of the disulfide bondsand thus depending on its position, the scrambling may or may not occur.

Plasmids below were constructed to find the effects on dimerization when uncoupled ~~Cyste~~cysteines are in different positions from the previous examples.

pMH42;B3(Fd)-SKPSISTKAS$(GGGGS)_2$GGGGC$(GGGGS)_3$GGPE-PE38; Ext(25CL19FA18)

pMH44;B3(Fd)-SKPSISTKAS$(GGGGS)_4$GGGGC$(GGGGS)_3$GGPE-PE38; Ext(35CL19FA18)

pMH46;B3(Fd)-SKPSISTKAS$(GGGGS)_6$GGGGC$(GGGGS)_3$GGPE-PE38; Ext(45CL19FA18)

The protein chains above have a fixed LFA sequence of L19FA18 in Ext(LFA) and the position of Cys is changed to the 25th, 35th or 45th. It is

designed to determine whether there are any limits on the position of ~~Cyste~~cysteine compared to dimers formed previously. When the uncoupled Cys was at the 45th position, detection of dimers was possible but not enough for purification. Accordingly, this example shows that dimerization is impossible if the uncoupled cysteine is located past the 45th position.

(Apparatus and method)

The same apparatus and method was used as example 1.

(Materials and Methods)

The same materials and methods were used as example 1.

(Construction of plasmids)

Protein expression plasmids were constructed by using primers and PCR. Plasmids for templates used were the same as in example 2 and example 6. For the repeated GGGGS sequence insertion, restriction and ligation was performed using restriction enzymes and ligase.

(Protein expression and isolation of inclusion body)

The same method was used as example 1.

(Refolding procedure and isolation of protein)

The same method was used as example 1.

(Construction of expression vector and isolation of inclusion body)

The cloned nucleotide sequence into expression plasmids made by PCR was confirmed by sequence analysis.

The proteins in the form of inclusion body were analyzed by

densitrometry analysis(TINA2.0) on PAGE gels. The ratio of desired product was 21~27% .

(Refolding of [B3(Fab)-Ext(10XC L19FA18)-PE38]$_2$) L19FA18

The production yield of dimer was calculated by the analysis of the samples with PAGE after refolding and ion exchange chromatography steps. The compared dimer production yield is shown on table 12.

Table 12

Plasmid name	Structure of Ext	Yield of dimer (%)	Position of Cys in Ext	Length of L(distance bet. Cys and F)	Number of GASQEND in L	Note
pCE1	Ext(1CL13FA7)	0.016	1	13	7	Example3
pMH21	Ext(4CL15FA11)	0.18	4	15	11	Example1
pMH22	Ext(4CL20FA16)	0.23	4	20	16	Example1
pCW1	Ext(15CL14FA13)	0.06	15	14	13	Example2
pMH42	Ext(25CL19FA18)	0.12	25	19	18	
pMH44	Ext(35CL19FA18)	0.04	35	19	18	
pMH46	Ext(45CL19FA18)	<<0.01	45	19	18	

According to table 12, when the ~~Cystec~~ysteine is at the 1st position (Ext(1CL13FA7)) the productivity is the lowest and when it is at the 4th position (Ext(4CL20FA16)), the productivity is the highest. When the Cys is positioned at the farthest (Ext(45CL19FA18)) position, the dimer can be detected but cannot be purified because the quantity is too small. If the protein production technology develops, it may become feasible.

This example shows there is no definite relation between the position of Cys on extension chain and the production yield. Also it shows that dimerization is possible when the uncoupled Cys position is within a certain range. This means that a Cys residue located past the 45th position is impractical for producing

dimers.

(Purification of [B3(Fab)-Ext(10XC L19FA18)-PE38]$_2$)

The samples were analyzed the same as example 1 and the differences in purity were confirmed with PAGE.

Abstract

The present invention relates to a method producing dimer of chimeric recombinant binding domain-heterogeneous functional group fusion([B-F fusion]$_2$) by using covalent disulfide-bond-bridge connecting the two monomers of chimeric recombinant binding domain-heterogeneous functional group fusion(B-F fusion). The dimer of chimeric recombinant binding domain(B)-heterogeneous functional group(F) fusion was the first to be formed by using covalent disulfide-bond-bridge to connect monomers to have double binding valency of the monomer. It has higher functional efficiency to its targets and the production yield is high by containing said extension peptide chain.